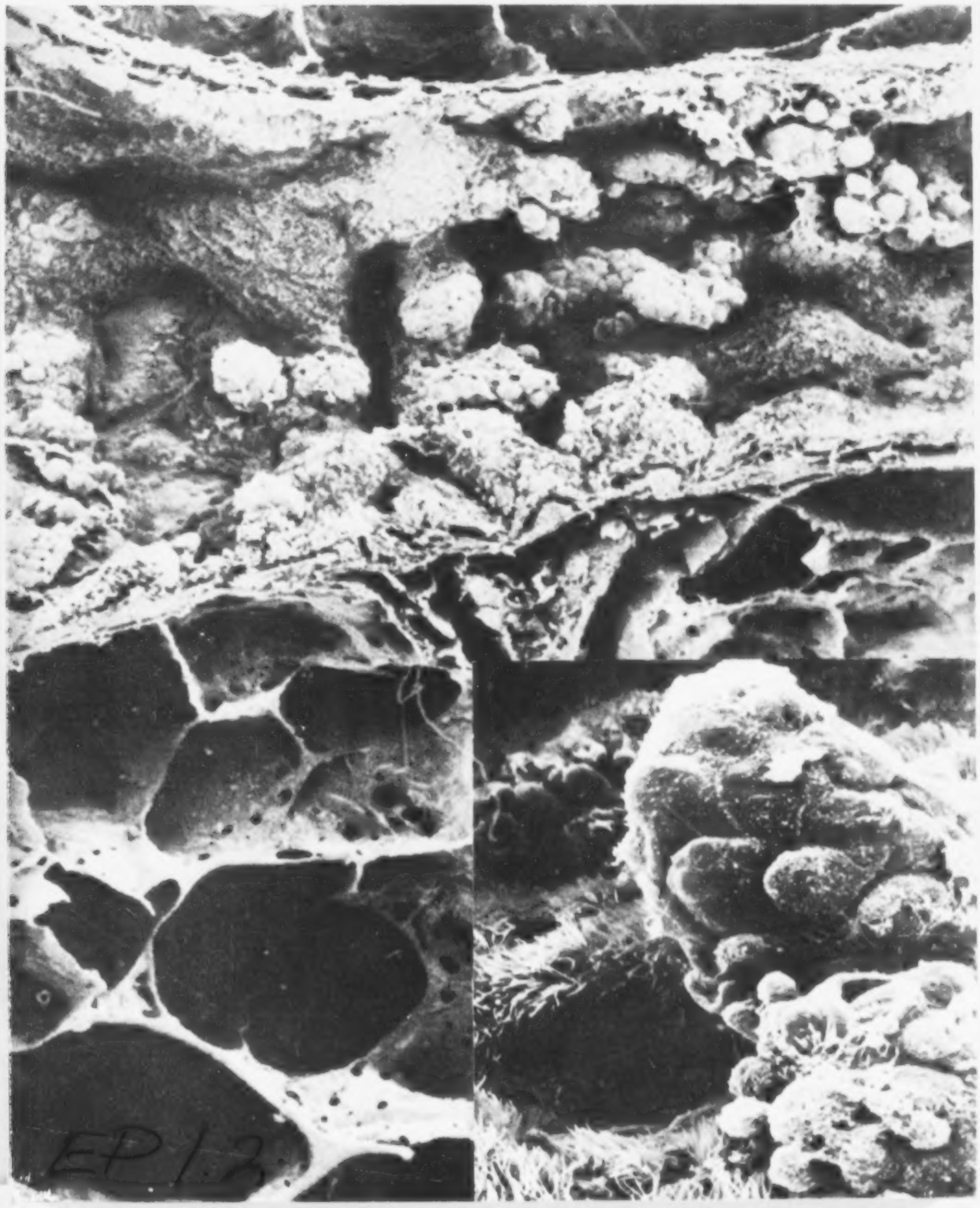
A; 7/40

United States
Environmental Protection
Agency

Environmental
Criteria and
Assessment Office

EPA-600/8-80-014

EPA Long-Term Effects of Air Pollutants: in Canine Species



EP 1.2

EPA-600 / 8-80-014
July, 1980

Long-Term Effects of Air Pollutants:

in Canine Species

NEWARK PUBL'C LIBRARY

OCT 24 1980

U. S. GOVT. PUBS.
DEPOSITORY 376

Editors:
J. F. Stara
D. L. Dungworth*
J. G. Orthoefer
W. S. Tyler*

Environmental Criteria and Assessment Office
U.S. Environmental Protection Agency
Cincinnati, Ohio

and

*Department of Pathology
School of Veterinary Medicine
University of California
Davis, California

DISCLAIMER

This report has been reviewed by the Environmental Criteria and Assessment Office, U.S. Environmental Protection Agency, Cincinnati, Ohio, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

The Clean Air Act of 1970 as amended in 1977 requires that a comprehensive data base be established to assess human health effects caused by air pollution from mobile sources. The spectrum of potential toxic effects can be viewed from two perspectives: The first is the identification of toxic effects from *combined* pollutants contained in mobile emissions; the second is detailing the long-term, low-level effects of the *individual* major ambient air pollutants, which are combustion by-products of automotive exhaust. Ideally, the major components of a data base used to develop health risk assessments are well-designed epidemiological studies and long-term, low-level animal studies. In epidemiological studies, the obvious limitation is the identification of the concentrations to which the specified population was exposed. Acute animal studies, although pertinent in some situations, cannot provide the information necessary for evaluating the potential hazard of the continuous, low-level exposure which is often more representative of existing environmental conditions. Chronic studies frequently establish effect levels below those used in acute studies, and provide the opportunity to assess irreversible tissue and organ changes which are often the major toxic manifestations of low-level insult.

The 9-year study presented in this monograph reviews the effects following exposure of dogs for 68 months to automotive exhaust, simulated smog, oxides of nitrogen, oxides of sulfur, and their combinations. Studies using canine species over extended periods of time have proven useful in the evaluation of risk to humans, especially when combined with epidemiological studies and human clinical investigations.

All of the data were reviewed by invited expert scientists at a conference held at Asilomar, California. Their evaluations and judgments form a significant segment of this monograph.

This research demonstrates a close alliance between government and university laboratories. For the first 6 years, the study was performed at the Environmental Toxicology Research Laboratory, U.S. Environmental Protection Agency, in Cincinnati. The concluding 3 years of the study were completed at the University of California, Davis. The collaborative effort between scientists of both institutions has produced data which will be a valuable contribution in the quest for a better understanding of the effects of mobile source pollution.

Stephen J Gage

Stephen J. Gage
Assistant Administrator
Office of Research
and Development, U.S. EPA

EXECUTIVE SUMMARY

Female beagle dogs comprising one control group (20) and seven treatment groups (12 each) were exposed 16 hours daily for 68 months to filtered air, raw or photochemically reacted auto exhaust, oxides of sulfur or nitrogen, or their combination. Mixtures of pollutants were chosen to provide various combinations of components generated by automobile emissions. The concentrations chosen were mostly in the approximate range of two to six times greater than averaged daily maxima occurring in several large cities during the exposure period. Specific exposures were to clean filtered air (CA), raw auto exhaust (R), irradiated auto exhaust (I), sulfur oxides (SO_x: 1100 $\mu g/m^3$ SO_2 and 90 $\mu g/m^3$ H_2SO_4), raw auto exhaust with added sulfur oxides (R + SO_x), irradiated auto exhaust with sulfur oxides (I + SO_x), and two mixtures of oxides of nitrogen — a high nitrogen dioxide level ($NO_L + NO_{2H}$: 1210 $\mu g/m^3$ NO_2 and 310 $\mu g/m^3$ NO) and a low nitrogen dioxide level ($NO_H + NO_{2L}$: 270 $\mu g/m^3$ NO_2 and 2050 $\mu g/m^3$ NO). Following the end of the 5-year exposure period, the dogs were kept for approximately 3 years in a normal indoor atmosphere to determine whether their health status would improve or worsen.

Clinical examinations were conducted regularly throughout the 8½-year experimental period. Hematologic studies were done at 6-month intervals. Special clinical examinations were: pulmonary function studies after 18, 36, and 61 months of exposure, and again 24 months after exposure ceased; cardiovascular studies after 48 and 54 months of exposure, and immediately prior to necropsy 32 to 36 months after the end of exposure; and neurologic assessment (visual evoked brain potentials) in the last month of exposure. Radiologic examinations were done 18 and 24 months after the end of exposure. Morphologic studies were based on necropsies performed 32 to 36 months after the end of exposure, though 16 pulmonary biopsy specimens obtained from control and two treatment groups were also examined. Samples of lung and bone were obtained at necropsy for subsequent collagen and lead determinations, and samples of brain, liver, heart, and lungs were obtained for lipid analysis.

The statistically significant and important findings were exposure-related abnormalities in pulmonary function and structure in the beagles. There was a good level of correlation between functional and structural abnormalities using pooled data of grouped animals. Current further analysis indicates good correlation on an individual dog basis. This correlation between functional and structural findings strengthens the validity and implications of the data.

Small abnormalities in pulmonary function were detected after 61 months of exposure, 7 months before it ended. When the same dogs were studied 2 years after the termination of exposure, many of their pulmonary function values showed an even greater difference from those of controls. The controls appeared to have stable pulmonary function values similar to those of other healthy beagles. Since these dogs were handled the same except for exposure and were of the same age and breed, it can be concluded that the differences measured were due to the effects of the exposure regimens. Exposed beagles had abnormalities in ventilatory, resistance, blood-gas exchange, and lung volume values. Groups exposed to R and R + SO_x had the greatest changes in indicators of airway disease — increases in pulmonary resistance, physiological dead space, and pulmonary arterial carbon dioxide. This finding was in accordance with subsequent morphologic observations. Groups exposed to SO_x and NO_2 had the greatest changes indicating pulmonary parenchymal abnormality — increases in lung volumes and dynamic compliance and decrease in pulmonary diffusing capacity. This finding also correlated well with the fact that these groups were most affected by early emphysematous-type lesions.

Two important structural abnormalities were present in lungs of exposed dogs. Morphologic and morphometric analysis revealed enlargement and breakdown of air spaces centered on respiratory bronchioles and alveolar ducts in all but the groups exposed to R and R + SO_x. It was most severe in the dogs exposed to high NO_2 or SO_x. The abnormality in air spaces can be considered to be analogous to early human centrilobular (centriacinar) emphysema. Although mild compared to clinically significant emphysema in man, it was sufficient to cause abnormalities in pulmonary function. The other major structural abnormality was hyperplasia of bronchiolar epithelial cells. It occurred to some degrees in all exposure groups but was worse in the dogs exposed to R and R + SO_x — the two groups lacking emphysematous-type changes.

Several important implications are raised by this study relative to assessment of the risks of air pollution on human populations and the establishment of air quality criteria. Mild but unequivocal functional and structural changes were produced in the lungs of beagles exposed to several mixes of pollutant at concentrations in the range of two to six times average daily maxima in selected cities. Taking high NO_2 as an example, the exposure concentration was 1210 $\mu g/m^3$ to which was added 310 $\mu g/m^3$ of the less toxic NO. In severe pollution episodes, peaks of NO_2 can exceed 300 $\mu g/m^3$. The National Ambient Air Quality Standard for NO_2 is an annual arithmetic mean 24-hour value of 100 $\mu g/m^3$. This is approximately one-tenth of the level that proved damaging to beagle lungs in the 68-month, 16-hour-a-day exposure. Similarly, with the SO_x exposure the levels were 1100 $\mu g/m^3$ of SO_2 and 90 $\mu g/m^3$ of H_2SO_4. Although the situation is compounded by the role of particulates and by difficulties in determining the exact states of sulfur oxides, SO_2 concentrations as such can exceed 250 $\mu g/m^3$ in severe pollution episodes. The air quality standard is an annual arithmetic mean 24-hour value of 80 $\mu g/m^3$. This is slightly less than one-tenth of the level causing pulmonary damage in the exposed beagles.

The structural abnormalities persisted for 3 years following the end of the 68-month exposure. The indication from the continued loss of pulmonary function after exposure ceased is that the damage worsened. The conclusion that the long-term, low-level exposures produced persistent damage, which although slight appeared to be progressive, makes the findings even more important in predicting possible effects in man over a life span. They provide strong support for no relaxation in the air quality standards.

A further conclusion is that because of the complexity of biological interactions in the lungs of an exposed man or animal, judiciously selected long-term studies will be necessary to fill the gap between short-term *in vitro* or *in vivo* experimental studies and epidemiological observations of human populations.

ACKNOWLEDGEMENTS

This research encompassed a period of over 9 years; during this time it was conducted uninterrupted at both the U.S. Environmental Protection Agency research laboratories in Cincinnati and the University of California, Davis. The editors acknowledge the assistance of all who were involved in the project: this includes scientists who have contributed to the study design and collection of data, and technical personnel who were involved in the daily operation of the exposure system, the animal chamber maintenance, animal care, physical and biological measurements and other research requirements. A special thanks goes to Dr. F. Gordon Hueter, presently the Director of the Health Effects Research Laboratory-RTP, Research Triangle Park, North Carolina, who initiated the study in 1965. Furthermore, the editors would like to acknowledge the individuals who compiled and reduced the enormous amount of data into an acceptable form.

In order to review and evaluate the data, a conference was held at Asilomar, California. Many individuals contributed to its technical and organizational success, and they have our appreciation. Special recognition is extended to: Carol Haynes (U.S. EPA) and Arlene Kasmire (UC, Davis), who made arrangements for the conference and speakers; and Dotty Morrissey (UC, Davis), Verna Tilford (U.S. EPA), and Donna Consiglio (UC, Davis), who typed and transcribed the proceedings. Their dedicated help made this monograph possible.

We also wish to express our thanks to Jan Connery of Energy Resources Co., Inc., who spent many long hours correcting and editing this monograph.

TABLE OF CONTENTS

Page

FOREWORD iii

EXECUTIVE SUMMARY iv

ACKNOWLEDGEMENTS vi

LIST OF FIGURES ix

LIST OF TABLES xiii

INTRODUCTION 1
J.F. Stara

CHAPTER

1. Study Overview, Rationale, Experimental Design, Experimental Facilities and Exposure Atmospheres 11
J.F. Stara, K. Busch, R.H. Hinners and J.K. Burkart

2. Exposure Chamber Atmospheres. Sampling and Analysis 41
M. Malanchuk

3. Clinical Summary of Beagle Study. Physical Examinations, Ocular Examinations, Hematology Examination (Post-Exposure) and Blood Chemistries 55
J.G. Orthoefer, J.F. Stara, Y.Y. Yang and K.I. Campbell

4. Collagen and Prolyl Hydroxlyase Levels in Lungs in Dogs Exposed to Automobile Exhaust and Other Noxious Gas Mixtures 71
R.S. Bhatnagar

5. The Effects of Air Pollutants on Membrane Lipids 87
G. Rouser and R. Aloia

6. Pulmonary and Cardiovascular Physiology Studies During Exposure 97
T.R. Lewis and W.J. Moorman

7. Review of the Cardiovascular and Pulmonary Function Studies on Beagles Exposed for 68 Months to Auto Exhaust and Other Air Pollutants 115
J.R. Gillespie

8. Review of Radiologic Studies 155
D. Dungworth

9. Effects of Air Pollutants on Visual Evoked Brain Potentials in Chronically Exposed Beagle Dogs 167
B.L. Johnson, J.G. Orthoefer and J.F. Stara

10. Ultrastructure of Biopsied Tissue 179
R.J. Stephens and G. Freeman

11. Necropsy 183
J.G. Orthoefer

12. Morphometric and Morphologic Evaluation of Pulmonary Lesions in Beagle Dogs Chronically Exposed to High Ambient Levels of Air Pollutants 195
D. Hyde, J.G. Orthoefer, D. Dungworth, W. Tyler, R. Carter and H. Lum

13. Critical Review. Implications and Plans for the Future 263
W. Tyler, D. Dungworth and J.F. Stara

LIST OF FIGURES

Page

FRONT COVER

Scanning electron micrograph of bronchiolar epithelial cell hyperplasia partially occluding a distal terminal bronchiole.

INTRODUCTION

Figure 1. Schedule of biological testing from 1965 to 1974 5

CHAPTER

1. Study Overview, Rationale, Experimental Design, Experimental Facilities and Exposure Atmospheres

Figure 1. Example of experimental animal — 15-inch beagle, female 12
Figure 2. Study time line 15
Figure 3. Inhalation chamber with placement of experimental animals 16
Figure 4. Five-year summary of experimental atmospheric levels 19
Figure 5. Engine room with the dynamometer system 24
Figure 6. Chronic auto exhaust study diagram 25
Figure 7. Irradiation chamber 26
Figure 8. Exposure chamber supply and exhaust flow diagram 27
Figure 9. System schematic diagram 29
Figure 10. Animal exposure chambers 33
Figure 11. Animal exposure chamber — Schematic diagram 34

2. Exposure Chamber Atmospheres. Sampling and Analysis

Figure 1. Pattern of differences in mean CAE study lead concentrations 48

3. Clinical Summary of Beagle Study. Physical Examinations, Ocular Examinations, Hematology Examination (Post-Exposure) and Blood Chemistries

Figure 1. Prevalence of chronic dermatitis in experimental animals 56
Figure 2. Prevalence of epiphora in experimental animals 57
Figure 3. The effect of exposure to auto exhaust on blood indices in the beagle dogs pre-exposure and exposure 59
Figure 4. The effect of exposure to auto exhaust on blood indices in the beagle dogs post-exposure 60

7. Review of the Cardiovascular and Pulmonary Function Studies on Beagles Exposed for 68 Months to Auto Exhaust and Other Air Pollutants

Figure 1. Mean values with standard errors for respiratory (rs), pulmonary (p), and chest wall (cw) resistances for each group of beagles 135

Figure 2. Mean values with standard errors for total lung capacity, functional residual volume, and residual volume (top to bottom of each bar) for each group of beagles 137
Figure 3. Mean dynamic compliance values (C_{dyn}) at different breathing frequencies for each group of beagles identified on the left of each C_{dyn}/f plot 137
Figure 4. Changes in each dog's residual volume (RV) between termination of exposure (TE) and two years after termination of exposure (2YR). 140
Figure 5. Changes in each dog's inspiratory lung compliance (C_L) between termination of exposure (TE) and two years after termination of exposure (2YR). 141

9. Effects of Air Pollutants on Visual Evoked Brain Potentials in Chronically Exposed Beagle Dogs

Figure 1. Typical averaged visual brain potential (VER) from a control treatment beagle dog 171
Figure 2. Means and standard errors for VER component N_1 latency versus air pollution treatment. 172
Figure 3. Means and standard errors for VER component P_1 latency versus air pollution treatment. 173
Figure 4. Means and standard errors for VER component N_1 amplitude versus air pollution treatment 174
Figure 5. Means and standard errors for VER component P_1 amplitude versus air pollution treatment 175
Figure 6. Means and standard errors for VER overall amplitude (RMS) versus air pollution treatment 176

11. Necropsy

Figure 1. Fixation of the right lung was accomplished using a dilute Karnovsky's fixture at 30 cm H_20 pressure for 16 to 18 hours 183

12. Morphometric and Morphologic Evaluation of Pulmonary Lesions in Beagle Dogs Chronically Exposed to High Ambient Levels of Air Pollutants

Figure 1. Cranial, middle, and caudal lobes of the canine right lung are depicted from the lateral view; the accessory lobe is depicted from the caudal view. Blocks of tissue for histologic evaluation were selected from indicated sites in transverse plane for cranial and middle lobes, sagittal plane for caudal lobe, and frontal plane for accessory lobe. 198
Figure 2. a. Pulmonary acinus from a control dog originates from a distal bronchus (b) and consists of a terminal bronchiole (Tb) with a nonuniform branching pattern of respiratory bronchioles (Rb) and alveolar ducts. b. Lung from the NO_2-high group of dogs at the same magnification illustrates distal air space enlargement and strands of tissue (arrow). 204

Figure 3. Comparison between the control and all other exposed groups by surface density (S_{va}, m^2/cm^3) and internal surface area of the right lung (S_{ar}, m^2). The control group to the left and all other exposed groups from left to right are plotted by decreases in S_{va}. Each bar represents the group mean ±1 standard error. Significant differences from the control groups are marked by an asterisk. 206

Figure 4. Comparison of distal airways and parenchyma in lungs from control and exposed groups. a. Numerous respiratory bronchioles (Rb) and alveolar ducts (Ad) are interspersed among alveoli in a lung from the control group. b. Slight enlargement of respiratory bronchioles (Rb) and alveolar ducts (Ad) of a representative lung from the NO-high group. c. Mild enlargement and loss of interalveolar septa in alveolar ducts (Ad) in a representative lung from the SO_x group. d. Mild to severe enlargement and loss of interalveolar septa characteristic of a lung from the NO_2-high group. A respiratory bronchiole (Rb) is adjacent to a greatly enlarged alveolar duct (Ad) 207

Figure 5. Comparison of distal airways in lungs from control and exposed dogs. a. Respiratory bronchiole (Rb) branches into alveolar ducts (Ad). Numerous alveoli and a moderate number of alveolar pores (arrow) are present in this lung of a control dog. b. Lung from the SO_x group of dogs at the same magnification illustrates alveolar duct (Ad) enlargement and loss of interalveolar septa. It is similar to the lesion in Figure 4c. c. Higher magnification in a region of b shows short interalveolar septa (black arrow) and shallow alveoli (a). Few interalveolar pores are seen (white arrow). 208

Figure 6. Respiratory bronchiole (Rb), alveolar ducts (Ad), and alveoli have the same conformation as controls despite widespread increase in interalveolar pores (inset, arrow) in a lung from the R group of dogs.. 209

Figure 7. Junction between a distal respiratory bronchiole (Rb) and proximal alveolar duct (Ad) shows air space enlargement with numerous alveolar pores and fenestrations in a lung from the NO_2-high group of dogs. Fenestrations and trabeculae (arrow) are common in alveolar ducts (inset). 211

Figure 8. Irregular hyperplasia of nonciliated bronchiolar cells (inset) partially occludes the lumen of a terminal bronchiole in a lung from the R group of dogs. An aggregation of inflammatory cells (arrow) is seen in the distal region of the terminal bronchiole.. 212

Figure 9. Comparison of terminal bronchiolar epithelium in lungs from control and exposed dogs. a. Epithelium of a terminal bronchiole (Tb) from the lung of a control dog has a mixture of ciliated (arrows) and nonciliated cells. b. Hyperplastic nodules in a terminal bronchiole (Tb) of a lung from the I group of dogs show a mixture of hypertrophied ciliated and nonciliated cells. Goblet cells (arrow) are also visible (1 μm Epon-embedded section). 213

Figure 10. Hyperplastic nodules composed primarily of nonciliated cells protrudes into the lumen of a terminal bronchiole (Tb) in a lung from the I group of dogs. The clear areas in the nonciliated cells (NC) represent areas of fixative-leached glycogen. A macrophage (M) and segments of a connective tissue core (arrows) are also present. An alveolar macrophage (AM) is in the airway lumen. 214

Figure 11. In a lung from the NO_2-high group of dogs, a ciliated cell (C) lies deep within a bronchiolar hyperplastic nodule, close to the basal lamina (bl). The cytoplasm has dispersed basal bodies (bb) and cilia are in the intercellular space (arrow). Serial sections showed cilia exiting from this cell. 216

Figure 12. In the dorsal membraneous portion of the trachea, the ciliated cells with short cilia (C) and long microvilli and the apical portion of goblet cells (G) are easily seen in a lung from a control dog. 217

Figure 13. Focus of ciliary loss without squamous metaplasia in a primary bronchus from a lung in the NO_2-high group of dogs. Ciliated cells are reduced in number and have fewer cilia per cell. Goblet cells do not have apical projections (G), but ciliated cells have numerous microvilli (C). 218

Figure 14. Focus of ciliary loss accompanied by squamous metaplasia is seen as a proliferative lesion (P) which protrudes into an airway lumen of a bronchus of a lung from the R group of dogs. The surface of the lesion has numerous microvilli and surface folds (inset). 219

Figure 15. Section of a proliferative lesion (P) that protrudes into the lumen (L) of a distal bronchus in a lung from the NO_2-high group of dogs illustrating squamous metaplasia. At least five cells deep, the lesion has a connective tissue core (C) containing numerous mononuclear inflammatory cells. (1 μm Epon-embedded section). 220

Figure 16. Thin section adjacent to the area seen in Figure 15. Columnar- to spindle-shaped cells with intracellular fibrils (F) and numerous intercellular processes connected by desmosomes (D) (inset) were the distinguishing characteristics of squamous metaplasia. A ciliated cell (C) sends cilia into the bronchial lumen (L). 221

LIST OF TABLES

CHAPTER Page

1. Study Overview, Rationale, Experimental Design, Experimental Facilities and Exposure Atmospheres

Table 1. Random Distribution and Number of Experimental Animals Per Chamber 13
Table 2. Composition of Exposure Atmospheres and Abbreviations Used 17
Table 3. Estimated Exposure Concentrations 17
Table 4. Comparison of Pollutant Levels in Chronic Dog Study with Ambient Atmospheric Concentrations in Four Cities — 1966-1971 18
Table 5. Methods for Reducing Errors in Animal Exposure Experiments 20
Table 6. Analysis of Variance of Red Blood Cell Counts (RBC) on 96 Young Female Beagles 22

2. Exposure Chamber Atmospheres. Sampling and Analysis

Table 1. Major Atmosphere Components and Measurement Strategy 42
Table 2. Pollutants Measured Automatically and Continuously 42
Table 3. Pollutants Measured Periodically 43
Table 4. Pollutant Concentrations in the Exposure Chamber Atmospheres During the Chronic Exposure Study 44
Table 5. Hydrocarbon Concentrations in the Exposure Atmospheres at Equilibrium 45
Table 6. Phototape Sampler Transmission Values 46
Table 7. Anions Associated with Airborne Particulate 46
Table 8. Acrolein 46
Table 9. Formaldehyde Concentrations 46

3. Clinical Summary of Beagle Study. Physical Examinations, Ocular Examinations, Hematology Examination (Post-Exposure) and Blood Chemistries

Table 1. Tabulation of Ocular Lesions, as Reported by Dr. Wyman 58
Table 2. Certain Hematological Abnormalities in Beagles in Relation to Different Treatments 61
Table 3. Date and Cause of Pre-Euthanasia Deaths in the Chronic Auto Exhaust Study 62

4. Collagen and Prolyl Hydroxylase Levels in Lungs in Dogs Exposed to Automobile Exhaust and Other Noxious Gas Mixtures

Table 1. Analysis of Variance for Hydroxyproline/Ninhydrin 73

Table 2. Mean Hydroxyproline/Ninhydrin (x 10^{-4}) by Treatment and the Results of Duncan's Multiple Range Test 73
Table 3. Analysis of Variance for Prolyl Hydroxylase 74
Table 4. Mean Prolyl Hydroxylase (dpm/mg) by Treatment and the Result of Duncan's Multiple Range Test (a =0.05) 74

5. The Effects of Air Pollutants on Membrane Lipids

Table 1. Heart Lipids (Mean Values) 88
Table 2. Liver Lipids (Mean Values) 90
Table 3. Lung Lipids (Mean Values) 91

6. Pulmonary and Cardiovascular Physiology Studies During Exposure

Table 1. Means and Standard Deviations of Body Weight and Pulmonary Function Tests — 18 Months 99
Table 2. Pulmonary Function Means by Treatment and by Variable at 36 Months of Exposure 100
Table 3. Pulmonary Function Means by Treatment and by Variable at 61 Months of Exposure 102
Table 4. Summary of the Electrocardiographic Data 104
Table 5. Summary of Vectorcardiographic Data 104
Table 6. Mean Widened-QRS-Complex (W-QRS-C) Indices for Air Pollutant-Exposed Groups of Dogs 105
Table 7. Mean Canine Blood Viscosity (Centipoise) 106
Table 8. Average Carboxyhemoglobin and Methemoglobin Concentrations in Canine Blood 106

7. Review of the Cardiovascular and Pulmonary Function Studies on Beagles Exposed for 68 Months to Auto Exhaust and Other Air Pollutants

Table 1. Grading System for Electrocardiograms 120
Table 2. Some ECG Interpretation Grading Criteria 120
Table 3a. ECG, VCG, and Other Diagnoses, by Sampling Period 121
Table 3b. ECG, VCG, and Other Diagnoses, by Sampling Period 122
Table 3c. ECG, VCG, and Other Diagnoses, by Sampling Period 123
Table 4. Selected Blood Pressures 124
Table 5. Phonocardiographic and Physical Abnormalities 125
Table 6. Dog Illnesses During Observation Period 126
Table 7. Dog Deaths During Observation Period 127
Table 8. Average Hematocrit, Carboxyhemoglobin, and Methemoglobin Levels 128
Table 9. Cardiovascular Function of Each Group 3 Years After Termination of Exposure 129
Table 10. Mean Intravascular Pressures of Each Group 3 Years After Termination of Exposure 130

Table 11. Mean Values and Standard Errors of Peripheral Vascular Resistance (R_{per}), Pulmonary Vascular Resistance (R_{pv}) and Left Ventricular Work (W_{Lv}) for Each Group 3 Years after Termination of Exposure 131
Table 12. Body Weight (WT), Blood Gases (pHa) Breathing Frequency (f), Tidal Volume (V_T), Minute Volume ($\dot{V}_{min}$), Percent Dead Space (%V_D), Dead Space (V_D), and Dead Space Ventilation ($\dot{V}_D$) of Beagles 133
Table 13. Total Dead Space of Control Group Compared with Exposed Groups 2 Years After Termination of Exposure. 134
Table 14. Pulmonary Diffusion Capacity (D_{LCO}) and D_{LCO} to Total Lung Capacity (TLC) Ratio (D_{LCO}/TLC) for the Beagles. 136
Table 15. Inspiratory and Expiratory Lung and Chest Wall Quasistatic Compliance Values for the Beagles. 136
Table 16. Lung Volumes of the Beagles in the Control Group at the Termination of Exposure (TE) and 2 Years After Termination of Exposure (2YR) Compared with Values from a Similar Group of Beagles Previously Studied at UC-Davis (External Controls) 138
Table 17. Compliances of Lung ($C_{L_{I\&E}}$) and of Chest Wall (C_{cw}) of the Beagles in the Control Group at the Termination of Exposure (TE) and 2 Years after Termination of Exposure (2YR) Compared with Values from a Similar Group of Beagles Previously Studied at UC-Davis (External Controls). 138
Table 18. Diffusing Capacity (D_{LCO}), D_{LCO}/Total Lung Volume Ratio (D_{LCO}/TLC) and Total Pulmonary Resistance (R_{pul}) of the Beagles in the Control Group at the Termination of Exposure (TE) and 2 Years After the Termination of Exposure (2YR) Compared with Values from a Similar Group of Beagles Previously Studied at UC-Davis (External Controls). 138
Table 19. The Mean Pulmonary Function Values of the Beagles Immediately Following Termination of Exposure (TE) and 2 Years after Termination (2YR). 139
Table 20. Mean Total Lung Capacity (ml) of Each Group of Beagles at End of Exposure (TE, column 1), 2 Years After End of Exposure (2YR, column 2), Change in Volume Between TE and 2YR (column 3), Predicted Value from External Controls (column 4), and the Difference Between Predicted and 2YR (column 5) 142

11. Necropsy

Table 1. Total Body Weights and Organ Weights and Standard Deviations for 70 Beagle Dogs. 184
Table 2. The Frequency Distribution of Mammary Tumors for Beagles Exposed to Exhaust and Non-Exhaust Atmospheres. 185

Table 3. The Frequency Distribution of Mammary Tumors for the Beagle Exposed to Different Atmospheres. 185
Table 4. Mucoidal Degeneration of A-V Valves 186
Table 5. Skin Lesions and Papillomas. 186
Table 6. Lung Lesions 187
Table 7. Lead Analysis of Femurs from Dogs (Beagles) Exposed to Chronic Auto Exhaust 187

12. Morphometric and Morphologic Evaluation of Pulmonary Lesions in Beagle Dogs Chronically Exposed to High Ambient Levels of Air Pollutants

Table 1. Measured Pollutant Concentrations to Which Dogs Were Exposed 16 Hours a Day for 68 Months 197
Table 2. Morphometric Parameters for Dog Lungs from Control and Exposed Groups 200
Table 3. Grading System for Selected Pulmonary Histologic Lesions 201
Table 4. Grading System for Selected Pulmonary Lesions Evaluated by SEM 202
Table 5. Summary of Results for Morphometric Parameters. 205
Table 6. Summary of Results of Pulmonary Lesions Evaluated by SEM 210

INTRODUCTION

J. F. Stara

As a result of the increasing concern about the hazards of chemical and physical agents which pollute our environment, a vast amount of research has been initiated to assess their health effects in exposed populations (1, 2). Epidemiological studies have demonstrated that high air pollution levels generated from stationary/industrial sources and associated with increased levels of sulfur compounds and particulates have caused increased morbidity and mortality, particularly in sensitive populations, e.g., among people with chronic pulmonary or cardiac disease. At the same time the literature reveals that there is less convincing epidemiologic evidence linking increased incidence of disease with increased photochemical smog levels in which the principal compounds identified are oxidants. In support of the epidemiological findings, a large number of animal toxicologic studies have been carried out to identify the health effects of common ambient air pollutants. However, the great majority of these investigations have been short-term and have used pollutant concentrations many times those encountered in the urban or rural ambient atmosphere (3, 4). Since the major objective of the U.S. Environmental Protection Agency is to abate or control environmental pollution to levels of acceptable risk, research investigations must provide a sufficient body of data so that informed judgments concerning acceptable levels of pollution in the environment can be made.

There is general agreement that, due to the extremely large number of potentially toxic agents present in the environment, a satisfactory assessment program must utilize a carefully conceived and comprehensively designed evaluation system. The best approach thus far is the employment of bioassay screening techniques for ranking the toxic potential of the many pollutants, and thus reducing the volume of compounds to a manageable number for further *in vivo* toxicity testing. Data obtained from the bioassay screening programs, along with appropriate analytical procedures and monitoring of the effluents and emissions from pollution sources, are then used to select a greatly reduced number of compounds for detailed biochemical, physiological and pathological toxicity studies of intermediate dosage and duration. Finally, on the basis of this information, only a few of the most ubiquitous and potentially harmful pollutants are selected for long-term mammalian studies at levels actually found in the environment. In this step-wise fashion, a comprehensive and reliable toxicologic assessment is obtained from which a sound administrative decision-making process and abatement action program can be promulgated.

In this introduction, the contributions of long-term studies in large mammalian species are reviewed in terms of their importance as valid predictors of similar clinical manifestations of harmful effects in man (5). To demonstrate the dependability of results and to confirm that such data probably could not have been obtained in shorter-lived species such as rodents because of the latency period needed for development of harmful biological effects, dogs and other higher mammals have been successfully used in a number of investigations, particularly in the fields of radiation and pesticide effects and anti-cancer drug research. For example, in a study of effects of anti-cancer drugs in dogs and monkeys, it was confirmed that the large animal species, over the long run, "... served to alert the physician to a significant proportion of the total spectrum of drug effects, which were encountered later during the clinical use of a new anti-cancer compound."

Dougherty *et al.* (6) have found that the important biological end-points observed in dogs exposed to ^{226}Ra and ^{228}Ra are quite comparable to those observed in humans and that deposition patterns of ^{239}Pu in dogs more closely approximate distribution in humans than do deposition patterns in rats. Clarke and Bair (7) administered $^{239}PuO_2$ to dogs as an aerosol; death resulted from 55 to 855 days post-exposure. Pathological effects observed included severe fibrosis followed by alveolar cell, bronchiolar and squamous types of metaplasia. Goldman and Della Rosa (8) have reported on the dynamics of ^{90}Sr metabolism in dogs exposed from intra-uterine time to adulthood. Their results indicate that the long-term dynamics of ^{90}Sr metabolism are similar in some respects to that of ^{90}Sr in man. Mays *et al.* (9) investigated the comparative effectiveness of ^{226}Ra, ^{239}Pu, ^{228}Ba and ^{90}Sr exposure in dogs in the induction of osteosarcomas in an attempt to elucidate the mechanism of radiation-induced bone cancer. Dungworth *et al.* (10) have investigated a spectrum of myeloproliferative disorders in response to ^{90}Sr exposure in dogs in a series of experiments which they hope will elucidate the relationship between granulocytic leukemia and myelofibrosis with myeloid metaplasia in man. Bustad *et al.* (11) have investigated hematopoietic changes in dogs maintained on a ^{90}Sr diet (six dose levels from 0.03 to 12 μCi/day) for the first 1.5 years of their lives. A persistent dose-related leukocyte depression was noted at the highest two dose levels. Eleven dogs 1.3 to 5 years old, fed the two highest dose levels, exhibited a terminal anemia and myeloid leukemia. Leach *et al.* (12) subjected dogs, monkeys and rats to the inhalation of natural UO_2 dust at a concentration of 5 mg/m^3, 6 hours per day, 5 days per week for periods up to 5 years. The lung and tracheobronchial lymphonode data for dogs and monkeys in this study suggest that there may be a radiation hazard in these tissues at or below the recommended TLV. These dogs were exposed for periods up to 5 years with little evidence of injury; however, 2 to 6 years post-exposure a high percentage of the dogs developed pulmonary neoplasia (13). Hobbs *et al.* (14), in comparing the toxicity of inhaled $^{144}CeO_2$ in dogs, mice and hamsters, found much lower doses to be effective in reducing the lifespan of the dogs compared to that of rodents on a μCi/kg basis. The rationale suggested is that rodents consistently have a much shorter lung retention of inhaled, relatively insoluble particles than dogs. In another study, Hobbs *et al.* (15) were unable to demonstrate pulmonary neoplasms in hamsters exposed to lung burdens of $^{239}PuO_2$ equivalent to those shown to produce neoplasms in dogs 3 years post-exposure. It is suggested that the latent period for tumor development may not be proportional to lifespan, in which case hamsters may not live long enough to develop pulmonary neoplasms.

Dogs have also proven useful in the evaluation of risk from insult imposed by a variety of chemicals, particularly pesticides. One study which illustrates the potential value of long-term dog studies of pesticide toxicity in terms of their value in the assessment of human health effects is that of Deichmann *et al.* (16). In this study dogs were dosed for 14 months with DDT. The pesticide concentration in the blood and fat during this time was comparable to levels in healthy occupationally exposed workers. The only abnormality noted in the dogs was an increase in alkaline phosphatase activity. Attempts were made to breed experimental males with experimental females after the cessation of pesticide feeding. Among the effects noted were: suppressed mammary development and milk production, stillbirths in one-half of the deliveries and depression of weanling survival rate from the normal of 84% to 32%. Other studies in which dogs have proven useful include: Yeary (17) (ferrocome); Worden *et al.* (18) (lenacil); Gallo (19) (phosalone); Mollello *et al.* (20) (probucol); Wernick *et al.* (21) (hair dyes); Woodard *et al.* (22) (xanthan gum); Worden *et al.* (23) (gusathion); Smyth *et al.* (24) (1, 2, 6 hexanetriol); Smith and Case (25) (fluorocarbons); Worden *et al.* (26) (ronnel); Earl *et al.* (27)

(diazinon); Hansen *et al.* (28) (2, 4 dichlorophenoxyacetic acid); King *et al.* (29) (succistearin); Case *et al.* (30) (nefopam hydrochloride); Muacevic *et al.* (31) (bromophos); Jenkins *et al.* (32) (benzene, toluene, xylene, and cumene); and Lyon *et al.* (33) (acrolein).

Another area in which dogs have proven useful test subjects concerns the quantification of effects from cigarette smoking. Auerbach *et al.* (34) have reported elevated heart weight to body weight ratios in smoking dogs, as well as pulmonary fibrosis and emphysema, which were similar to conditions reported in humans. In addition, Auerbach *et al.* (35) have shown a thickening of myocardial arteriole walls in smoking dogs. Thickening increased with the duration of smoking and with the number of cigarettes smoked, and in dogs smoking nonfilter as opposed to filter cigarettes. These results parallel effects noted in humans. Hammond *et al.* (36) have found that the responses observed in dogs following smoking closely parallel those observed in man, in that the types of histologic changes produced in the lung parenchyma were the same in both species. In both species there was a dose-response relationship and the degree of damage to the lung parenchyma increased with increasing duration of cigarette smoking. Battista *et al.* (37) have found that following exposure to five cigarettes daily, dogs exhibited a 7% increase in central airway resistance when exposed to whole smoke and less than a 1% increase when exposed to the gas phase alone; a 30% increase in minute volume following whole smoke exposure and only a 1% increase following gas phase exposure; and a 5% decrease in peripheral airway resistance for both whole smoke and gas phase exposure.

Another large mammal which has been used extensively in long-term toxicity testing is the miniature swine. This animal has proven to be especially useful in the study of ^{90}Sr-induced leukemia. Clarke *et al.* (38), Clarke *et al.* (39), Howard and Clarke (40) and Ragan *et al.* (41) have published a series of reports which describe various aspects of a study in which miniature swine were dosed orally with ^{90}Sr at levels from 1 to 3100 μCi/day for 7 to 10 years. These reports indicate that bone marrow damage rather than osteosarcomas may be the limiting factor in chronic *oral* toxicity of ^{90}Sr and that the latent period for leukemia induction is considerably shorter than that for osteosarcoma development. In these studies the parental generation, which was exposed to ^{90}Sr at levels from 25 to 125 μCi/day from 9 months of age, exhibited primarily myeloproliferative disorders consisting of a progressive decrease in circulating leukocytes and platelets and a precipitous terminal fall in erythrocytes. A second syndrome was noted primarily in groups dosed at levels of 125 and 625 μCi/day. This syndrome was characterized by broad spectrum myeloproliferation ranging from myeloid metaplasia to frank blast cell leukemia. In contrast, the offspring demonstrated a marked increase in the incidence of lymphoproliferative disorders in addition to the myeloproliferations. In addition, ^{90}Sr, up to levels where females did not survive long enough to bear young, did not affect fetal or neonatal mortality.

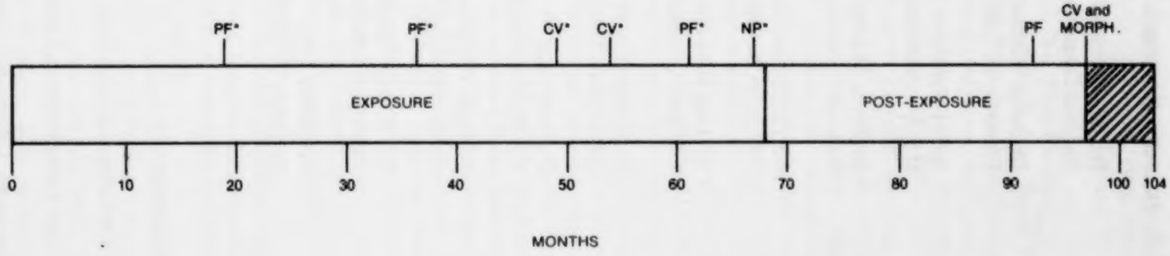
Primates constitute another group of animals frequently chosen for long-term toxicity studies when extrapolation to the human population is a goal. Examples of studies in which primates have proven useful include a series of experiments reported by Spalding *et al.* (42, 43, 44). These studies involve the effects of radiation exposure in primates with respect to dose protraction and residual injury and recovery rate expecially in relation to the concept of equivalent residual dose.

Another animal which has been used extensively and has proven to be quite useful in a number of long-term studies is the cat. Several papers which discuss a number of parameters relating to orally administered ^{89}Sr have been published by Berman and Stara (45), Nelson *et al.* (46), Stara and Wolfangel (47), Nelson *et al.* (48), and Rosenstein and Nelson (49). Topics discussed in these papers include: tissues uniquely sensitive to radiation-induced lymphoproliferative or myeloproliferative disorders during growth and development of the cat; assessment of the radiation dose rate delivered to the bone marrow and other organs in cats of different ages; and the relationship between the effective dose of ^{89}Sr in cats and the ^{90}Sr dose which is leukemogenic in dogs and miniature swine. In two more recent studies with dogs, Benjamin *et al.* (50) have shown differences in the cause of death depending on time following inhalation exposure to ^{144}Ce and ^{90}Sr. In dogs dying within 2 years of exposure, deaths were attributable to non-neoplastic radiation lesions, whereas in dogs dying later than 2 years following exposure, neoplasms were the primary cause of death. Norris *et al.* (51) are in the process of developing dose-response curves for dogs exposed to ^{60}Co (X&Y) radiation. Preliminary data indicate a shift in the response curve at low dosages so that injury is independent of dose rate and dependent only upon the total accumulated dose.

This monograph presents the results of a major study of the effects of air pollutants in beagles, a high mammalian species. The work was performed at the EPA's Health Effects Research Laboratory in Cincinnati, Ohio, from 1965 to 1971, after which the dogs were moved to the University of California at Davis for further study during the next three years. The primary objective of the study was to examine the effects of long-term exposure to environmentally realistic levels of whole automobile exhaust and its individual components. The irreversible chronic effects in dogs were assessed in order to provide valuable data on the potential toxic effects in humans.

The schedule of biological tests conducted during the investigation is shown in Figure 1. Results for the major parameters of interest (pulmonary, cardiovascular, hematological, and pathological) are presented in the chapters of this monograph, together with neurophysiologic (EEG) observations, clinical chemistry findings, assessment of immune competence, the results of continuous clinical observations of the animals, and other significant findings.

To assess the implications of these data, a special conference was organized exclusively to review and evaluate all aspects of the study. It was held at Asilomar, California, on August 25, 26 and 27, 1976, and attended by 21 scientists and academicians from government agencies, research institutes, and universities. The attendees and their affiliations are listed below. Portions of the discussions that took place at the Asilomar Conference are presented at the end of each chapter. Chapter 13, which consists solely of conference discussions, provides a detailed critical review of the study, and presents ideas for future studies of long-term, low-level pollutant effects in higher mammals. The work presented in the following chapters demonstrates the dire need for and the value of long-term animal toxicology data which parallel more closely the effects on man of chronic low-level exposures to environmental and occupational contaminants.



LEGEND: PF — PULMONARY FUNCTION STUDIES
CV — CARDIOVASCULAR STUDIES
MORPH — QUANTITATIVE MORPHOLOGY STUDIES
NP — NEUROPHYSIOLOGIC (EEG) STUDIES
* — DESIGNATES CINCINNATI STUDIES

NOTE: HEMATOLOGY EVERY SIX MONTHS

CONT. CLINICAL OBSERVATIONS AND TREATMENT (ESP. AFTER 9/69)

Figure 1
Schedule of biological testing from 1965 to 1974.

Asilomar Conference Discussion Participants

Dr. Roy Albert
U.S. Environmental Protection Agency

Dr. Joseph Brain
Harvard School of Public Health

Dr. Rajendra Bhatnagar
University of California

Mr. Kenneth Busch
National Institute of Occupational Safety and Health

Dr. Donald Dungworth
University of California

Dr. Jerry Gillespie
University of California

Dr. F. Gordon Hueter
U.S. Environmental Protection Agency

Dr. Dallas Hyde
University of Florida

Dr. Jerome Kleinerman
St. Luke's Hospital

Dr. Si Duk Lee
U.S. Environmental Protection Agency

Dr. Trent Lewis
National Institute of Occupational Safety and Health

Dr. Neil Littlefield
National Center for Toxicological Research

Dr. James MacEwen
University of California

Mr. Myron Malanchuk
U.S. Environmental Protection Agency

Dr. Bryon McLees
National Institutes of Health

Dr. Paul Nettesheim
Oak Ridge National Laboratory

Dr. John Orthoefer
U.S. Environmental Protection Agency

Dr. George Rouser
City of Hope National Medical Center

Dr. Jerry Stara
U.S. Environmental Protection Agency

Dr. Robert Stephens
Stanford Research Institute

Dr. Whitney Thurlbeck
University of Manitoba

Dr. Walter Tyler
University of California

References

1. Drew, R.T., and H. Witschi. 1976. Target organ toxicity: lung, introduction. Environ. Health Perspec. 16: 1-2.
2. Brain, J.D. 1977. The respiratory tract and the environment. Environ. Health Perspec. 20: 113-126.
3. MacFarland, H.N. 1975. Inhalation toxicology. J. Assoc. Off. Anal. Chem. 58: 689-91.
4. MacFarland, H.N. 1976. Respiratory toxicology, essays in toxicology. Academic Press, Vol. 7: 121-154.
5. Krasovskii, G.N. 1976. Extrapolation of experimental data from animals to man. Environ. Health Perspec. 13: 51-58.

6. Dougherty, T.F., B.J. Stover, J.H. Dougherty, W.S.S. Jee, C.W. Mays, C.E. Rehfeld, W.R. Christensen, and H.C. Goldthorpe. 1962. Studies of the biological effects of ^{226}Ra, ^{239}Pu, ^{228}Ra (MsTh), ^{228}Th (RdTh) and ^{90}Sr in adult beagles. Radiation Res. 17: 625-81.
7. Clarke, W.J., and W.J. Bair. 1964. Plutonium inhalation studies — VI. Pathologic effects of inhaled plutonium particles in dogs. Health Physics 10: 391-398.
8. Goldman, M., and R.J. Della Rosa. 1967. Studies on the dynamics of strontium metabolism under conditions of continuous ingestion to maturity. Pp. 181-94 *in* J.M. Lenihan *et al.*, eds. Strontium metabolism. Academic Press.
9. Mays, C.W., T.F. Dougherty, G.N. Taylor, R.D. Lloyd, B.J. Stover, W.S.S. Jee, W.R. Christensen, J.H. Dougherty, and D.R. Atherton. 1969. Radiation-induced bone cancer in beagles. Pp. 387-408 *in* C.W. Mays, *et al.*, eds. Delayed effects of bone-seeking radionuclides. University of Utah Press, Salt Lake City.
10. Dungworth, D.L., M. Goldman, J.W. Switzer, and D.H. McKelvie. 1969. Development of a myeloproliferative disorder in beagles continuously exposed to ^{90}Sr. Blood 34: 610.
11. Bustad, L.K., M. Goldman, L.S. Rosenblatt, D.H. McKelvie, and I.I. Hertzendorf. 1969. Hematopoietic changes in beagles fed ^{90}Sr. *In* C.W. Mays, *et al.*, eds. Delayed effects of bone-seeking radionuclides. University of Utah Press, Salt Lake City.
12. Leach, L.J., E.A. Maynard, H.G. Hodge, J.K. Scott, C.L. Yule, G.E. Sylvester, and H.B. Wilson. 1970. A five-year inhalation study with natural uranium dioxide dust (UO_2). I. Retention and biologic effect in the monkey, dog and rat. Health Physics 13: 559-612.
13. Leach, L.J., C.L. Yulie, H.C. Hodge, G.E. Sylvester, and H.B. Wilson. 1973. A five-year inhalation study with natural uranium oxide dust. II. Post-exposure retention and biological effects in the dog and rat. Health Physics 25: 239-58.
14. Hobbs, C.H., R.O. McClellen, and S.A. Benjamin. 1974. Toxicity of inhaled $^{144}CeO_2$ in immature, young adult and aged Syrian hamsters. II. Inhalation Toxicol. Res. Instit. Ann. Report, 1973-1974, Lovelace Fn., Albuquerque, N.M.
15. Hobbs, C.H., J.A. Mewhinney, D.A. Slauson, R.O. McClellen, and J.J. Meglio. 1974. Toxicity of inhaled $^{239}PuO_2$ in immature, young adult and aged Syrian hamsters. II. Inhalation Toxicol. Res. Inst. Ann. Rep. 1973-1974, Lovelace Fn., Albuquerque, N.M.
16. Deichmann, W.B., W.E. MacDonald, A.G. Beasley, and D. Cubit. 1971. Subnormal reproduction in beagle dogs induced by DDT and aldrin. Ind. Med. Surg. 40: 10-20.
17. Yeary, R.A. 1969. Chronic toxicity of dicyclopentadienyliron (ferrocene) in dogs. Toxicol. Appl. Pharmacol. 15: 667-76.
18. Worden, A.M., P.R.B. Noel, L.E. Mawdesley-Thomas, A.K. Palmer, and M.A. Fletcher. 1974. Feeding studies on lenacil in the rat and dog. Toxicol. Appl. Pharmacol. 27: 215-24.
19. Gallo, M.A. 1976. Chronic toxicologic investigations of phosalone in the dog. Toxicol. Appl. Pharmacol. 36: 561-68.
20. Mollello, J.A., C.G. Gerbig, and V.B. Robinson. 1973. Toxicity of (4,4'-(isopropylindenedithio) bis (2,6-di-t-butylphenol)), probucol, in mice, rats, dogs and monkeys: demonstration of a species specific phenomenon. Toxicol. Appl. Pharmacol. 24: 59-93.
21. Wernick, T., B.M. Lanman, and J.L. Fraux. 1975. Chronic toxicity, teratologic and production studies with hair dyes. Toxicol. Appl. Pharmacol. 32: 450-60.
22. Woodard, G., M.W. Woodard, W.H. McNeely, P. Kovacs, and M.T.I. Cronin. 1973. Xanthan gum: safety evaluation by 2-year feeding studies in rats and dogs and a 3-generation reproduction study in rats. Toxicol. Appl. Pharmacol. 24: 30-36.
23. Worden, A.M., G.H. Wheldon, P.R.B. Noel, and L.E. Mawdesley-Thomas. 1973. Toxicity of gusathion for the rat and dog. Toxicol. Appl. Pharmacol. 24: 405-12

24. Smyth, H.F. Jr., U.C. Pozzani, C.S. Weil, M.J. Tallant, and C.P. Carpenter. 1969. Experimental toxicity and metabolism of 1, 2, 6 hexanetriol. Toxicol. Appl. Pharmacol. 15: 282-86.
25. Smith, J.K., and M.T. Case. 1973. Subacute chronic toxicity studies of fluorocarbon propellants in mice, rats and dogs. Toxicol. Appl. Pharmacol. 26: 438-443.
26. Worden, A.N., P.R.B. Noel, and L.E. Mawdesley-Thomas. 1972. Effect of ronnel after chronic feeding to dogs. Toxicol. Appl. Pharmacol. 23: 1-9.
27. Earl, F.L., B.E. Melveger, J.E. Reinwall, G.W. Bierbower, and J.M. Curtis. 1971. Diazinon toxicity-comparative studies in dogs and miniature swine. Toxicol. Appl. Pharmacol. 18: 285-95.
28. Hansen, W.H., M.L. Quaife, R.T. Habermann, and O.G. Fitzhugh. 1971. Chronic toxicity of 2, 4-dichlorophenoxyacetic acid in rats. Toxicol. Appl. Pharmacol. 20: 122-29.
29. King, W.R., W.R. Michael, and R.H. Coots. 1971. Feeding of succistearin to rats. Toxicol. Appl. Pharmacol. 18: 26-34.
30. Case, M.T., J.K. Smith, and R.A. Nelson. 1976. Chronic oral toxicity studies of nefopam hydrochloride in rats and dogs. Toxicol. Appl. Pharmacol. 36: 301-306.
31. Muacevic, G., E. Dirks, H.J. Kinkel, and F. Leuschner. 1970. Anticholinesterase activity of bromophos in rats and dogs. Toxicol. Appl. Pharmacol. 16: 585-96.
32. Jenkins, L.J., Jr., R.A. Jones, and J. Siegel. 1970. Long-term inhalation screening studies of benzene, toluene, 0-xylene and cumene on experimental animals. Toxicol. Appl. Pharmacol. 16: 818-23.
33. Lyon, J.P., J. Jenkins, Jr., R.A. Jones, R.A. Coon, and J. Siegel. 1970. Repeated and continuous exposure of laboratory animals to acrolein. Toxicol. Appl. Pharmacol. 17: 726-732.
34. Auerbach, O., E.C. Hammond, D. Kirman, and L. 1967. Garfinkel. Emphysema produced in dogs by cigarette smoking. J.A.M.A. 199: 241-46.
35. Auerbach, O., E.C. Hammond, and L. Garfinkel. 1971. Thickness of walls of myocardial arterioles in relation to smoking and age. Arch. Environ. Health 22: 20-27.
36. Hammond, E.C., D. Kirman, and L. Garfinkel. 1970. Effects of cigarette smoking on dogs. Arch. Environ. Health 21: 740-53.
37. Battista, S.P., W.B. Steber, and C.J. Kensler. 1974. Pulmonary function in spontaneously breathing dogs with lower airway catheters. Arch. Environ. Health 28: 164.
38. Clarke, W.J., R.F. Palmer, E.B. Howard, and P.L. Hackett. 1970. Strontium-90: Effects of chronic ingestion on farrowing performance of miniature swine. Science 169: 598-600.
39. Clarke, W.J., E.B. Howard, and P.L. Hackett. 1969. Strontium-90 induced neoplasia in swine. Pp. 263-77 *in* C.W. Mays *et al.*, eds. Delayed effects of bone-seeking radionuclides. University of Utah Press, Salt Lake City.
40. Howard, E.B., and W.J. Clarke. 1970. Induction of hematopoietic neoplasms in miniature swine by chronic feeding of ^{90}Sr. J. Natl. Cancer Instit. 44: 21-38.
41. Ragan, H.A., P.L. Hackett, B.J. McClanhan, and W.J. Clarke. 1973. Pathologic effects of chronic ^{90}Sr ingestion in miniature swine. Pp. 919-929 *in* L.T. Harmison, ed. Research animals in medicine. DHEW Pub. No. (NIH) 72-333 GPO, Washington, D.C.
42. Spalding, J.F., L.M. Holland, O.S. Johnson and J.R. Prine. 1975. Recovery rate and residual injury in monkeys exposed to large doses of radiation by fractionation. Radiation Res. 62: 70-78.
43. Spalding, J.F., L.M. Holland, J.R. Prine, P.M. LaBauve, and J.E. London. 1973. Comparative effects of dose protraction and residual injury in dogs and monkeys. Radiation Res. 54: 453-62.

44. Spalding, J.F., L.M. Holland, and J.R. Prine. 1972. The effects of dose protraction on hematopoiesis in the primate and dog. Life Sciences and Space Research. X-Akademie-Verlag, Berlin.
45. Berman, E., and J.F. Stara. 1968. Qualitative evaluation of the embryonic skeleton in cats. Pp. 73-78 *in* Radiation Bioeffects Summary Report, Jan.-Dec. 1968, U.S. HEW.
46. Nelson, N.S., J.F. Stara, and R.C. Wolfangel. 1968. ^{85}Sr distribution and weights of organ systems in chronically-labelled cats. Pp. 70-72 *in* Radiation Bioeffects Summary Report, Jan.-Dec. 1968, U.S. HEW.
47. Stara, J.F., and R.G. Wolfangel. 1968. Metabolism of ^{85}Sr in cats. Pp. 66-69 *in* Radiation Bioeffects Summary Report, Jan.-Dec. 1968, U.S. HEW.
48. Nelson, N.W., R.G. Wolfangel, and J.F. Stara. 1968. Placental transport of strontium and calcium in the *Felis domestica*. Pp. 63-65 *in* Radiation Bioeffects Summary Report, Jan.-Dec. 1968, U.S. HEW.
49. Rosenstein, L.S., and N.S. Nelson. 1968. Dosimetry of radio-strontium in skeletal tissues of cats. Pp. 73-78 *in* Radiation Bioeffects Summary Report, Jan.-Dec. 1968, U.S. HEW.
50. Benjamin, S.A., F.F. Hahn, T.L. Chiffelle, B.B. Boecker, C.H. Hobbs, R.K. Jones, and M.B. Snipes. 1975. Occurrence of hemangiosarcomas in beagles with internally deposited radionuclides. Cancer Res. 35: 1745-55.
51. Norris, W.P., S.A. Tyler, and G.A. Sacher. 1975. An interspecies comparison of the response of mice and dogs to continuous ^{60}CoY-irradiation. IAEA Int. Symp. on Biological Effects of Low Level Radiation Pertinent to the Protection of Man and His Environment. Chicago, Illinois, Nov. 3-7, 1975.

1. STUDY OVERVIEW, RATIONALE, EXPERIMENTAL DESIGN, EXPERIMENTAL FACILITIES AND EXPOSURE ATMOSPHERES

J.F. Stara, K. Busch, R.G. Hinners and J.K. Burkart

Introduction

A long-term research study was conducted in order to obtain information necessary to elucidate the public health consequences of emissions from mobile and stationary sources and to provide an appropriate data base for air quality criteria development. Automotive exhaust and stationary source emissions are composed of a complex mixture of interacting chemical substances of varying toxicity. A number of the components which pose a potential hazard to human health have not yet been fully investigated, nor have their respective contributions to human illness been fully determined. While brief animal exposures provide useful information on acute toxicity of emission components, extended chronic exposures at realistic environmental levels are necessary to evaluate the long-term effects of air contaminants on biological systems. This introductory paper is intended to familiarize the reader with the study rationale and experimental design, including study duration, the complicated inhalation facility, the original pollutant mixtures used, and the various biological parameters measured.

The experimental facilities designed for inhalation toxicologic studies of complex air pollution mixtures were unique in 1965 and continue to be so. Currently our laboratory staff is involved in other inhalation studies investigating the health effects of emissions from diesel engines and from automotive pollution control systems such as the catalytic converter. The major individual pollutants implicated in the exhaust, e.g., sulfuric acids, sulfates, platinum, palladium and ruthenium, are currently under study. The system is unique because the auto exhaust, simulated smog and associated compounds are ducted to animal chambers for determination of toxic effects at carefully controlled levels.

The major objective of the study reported in this monograph was the identification and, if possible, the quantitation of the biological effects in dogs exposed to low-level automotive emissions and individual ubiquitous ambient air pollutants during the most active portion of their life spans. Of particular interest was the determination of possible irreversible effects caused by the tested pollutants. Young female beagles were exposed for 16 hours daily to raw and photochemically reacted auto exhaust, oxides of nitrogen, oxides of sulfur, and combinations thereof, for approximately 6 years. Thereafter, they were allowed to live for 3 years in a normal atmosphere in order to permit a recovery from transient effects.

Experimental Animals

Beagle dogs were selected as the experimental animals primarily because of their large size, which is well suited for various physiological measurements and repeated sampling, and because of their long life span, which permits a detailed observation of the incidence and progress of developing disease patterns (Figure 1). From the previous long-term studies in dogs reported in the introduction, it was felt that the progressive degenerative changes observed in dogs would closely parallel the slowly developing harmful effects of low-level pollutants in man. The use of smaller animals such as rodents, with their much shorter life span, would not adequately satisfy these requirements.

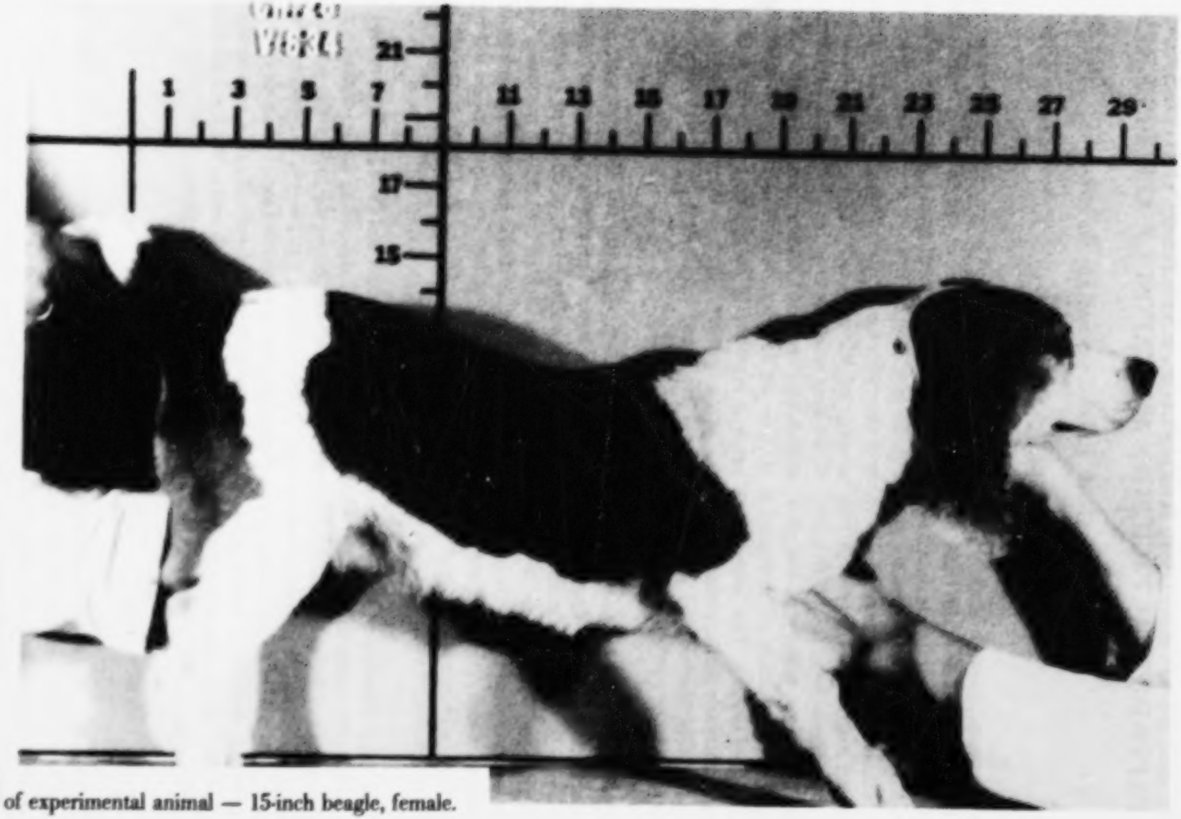

21
1
3
5
7
11
13
15
17
19
21
23
25
27
29
17
15

Figure 1
Example of experimental animal — 15-inch beagle, female.

One hundred and four (104) female beagles, approximately 4 months of age, were obtained from a similar genetic pool, and assembled into an experimental colony. After an environmental acclimatization period, the beagles were divided into seven groups of 12 animals and one group of 20, and placed into 26 inhalation chambers, with 4 animals per chamber. Animal group selections and chamber assignments were performed at random, i.e., a complete randomization was performed in the allocation of animals to specific exposure atmospheres and to the triplicate chambers for each atmosphere (See Table 1).

Table 1. Random Distribution and Number of Experimental Animals Per Chamber[a]

							Total no. of animals	
Atmosphere[b]							**Beginning of exposure**	**End of exposure**
(1) CA	X	#2	#9	#11	#21	#25		
	Y	4	4	4	4	4	20	17
	Z	4	2	4	3	4		
(2) R	X	#5	#12	#23				
	Y	4	4	4			12	10
	Z	3	4	3				
(3) I	X	#14	#16	#22				
	Y	4	4	4			12	10
	Z	4	4	2				
(4) SO_x	X	#4	#10	#20				
	Y	4	4	4			12	8
	Z	3	4	1				
(5) R + SO_x	X	#6	#18	#24				
	Y	4	4	4			12	10
	Z	4	4	2				
(6) I + SO_x	X	#7	#15	#17				
	Y	4	4	4			12	11
	Z	4	3	4				
(7) $NO_L + NO_{2H}$	X	#8	#19	#26				
	Y	4	4	4			12	10
	Z	2	4	4				
(8) $NO_H + NO_{2L}$	X	#3	#13	#27				
	Y	4	4	4			12	10
	Z	4	3	3				
						Totals	**104**	**86**

[a] Key: X = *Chamber number (selection was randomized).*
Y = *Number of dogs at outset of experiment.*
Z = *Number of surviving animals at the termination of exposure.*

[b] See Table 2 for precise composition of each exposure atmosphere.

Study Duration and Exposure Atmospheres

The study was initiated in the fall of 1965 (Figure 2). At the outset of the experiment three mammalian species, mice, guinea pigs and female beagle dogs, were placed into inhalation exposure chambers. The rodents were placed on the top shelf and the beagles on the lower shelf (Figure 3). The rodents were removed after a short period of exposure in order to study acute and subacute effects. The exposure of dogs was discontinued after a 68-month period, i.e., at the time when the animals were almost exactly 6 years of age. The post-exposure period of observations and testing lasted approximately 3 years, at which time the animals were euthanized and pathology studies were performed.

Two major reviews of this long-term investigation were performed by expert scientific committees. After 4 years of exposure, when the dogs were 50 months old, a scientific committee reviewed the data and recommended that additional tests be performed especially addressing the cardiovascular system. The committee felt that the study should be conducted over the life span of the animals in the animal inhalation chambers under a continuous exposure regimen (3). However, the study was ended in 1970 because the chamber space was needed for other research. Fortunately, an agreement was subsequently made to allow continuation of the animal observation and testing outside of the exposure chambers, i.e., in ambient air, for an additional period of time. An in-house committee decided in 1971 to contract for 3 years the maintenance, observation, and continuation of *in vivo* testing and final sacrifice of all animals with the Department of Pathology, University of California at Davis (5).

The exposure atmospheres consisted of a clean air control (CA) and seven experimental exposures as follow: nonirradiated auto exhaust (R), irradiated auto exhaust (I), oxides of sulfur ($SO_2+H_2SO_4$), two concentrations of oxides of nitrogen (NO-low + NO_2-high and NO-high + NO_2-low), and two mixtures of auto exhaust and oxides of sulfur ($R+SO_x$ and $I+SO_x$) (Table 2). The initial exposure levels of the various atmospheres are presented in Table 3. The intent in the study protocol (6) was to expose the dogs for a long period of time to realistic urban pollutant levels. Table 4 presents maximum 24-hour means, maximum annual point values and annual arithmetic means for ambient air pollutants in four highly industrialized urban communities during the 6 years of this study (1966-1971) (7, 8). Comparison to pollutant levels in animal chamber exposure atmospheres indicates that the levels to which the dogs were exposed were generally from two to six times as great as the reported maximum 24-hour means. In the case of SO_2 in Chicago during the years 1966, 1967, 1968 and 1969, exposure levels were directly comparable to the maximum 24-hour means. In addition, in certain years maximum yearly values for NO, NO_2 and oxidants were within the same range as experimental dog exposure levels. Although maximum 24-hour means and maximum annual values are not precisely representative of ambient levels over long periods, nevertheless these comparisons show that for various time intervals certain segments of the population have been exposed to levels of pollutants which approximate the levels used in this study for long-term exposure of beagles. For this reason, the authors feel that the study can be appropriately termed a "long-term exposure of dogs to elevated urban air pollution levels."

The 5-year summary of average measured chamber concentrations is presented in Figure 4. All exposure levels of the various pollutants show a rather steady trend, with the exception of nitrous oxide. The averages shown are over triplicate chambers for 4- to 6-month periods.

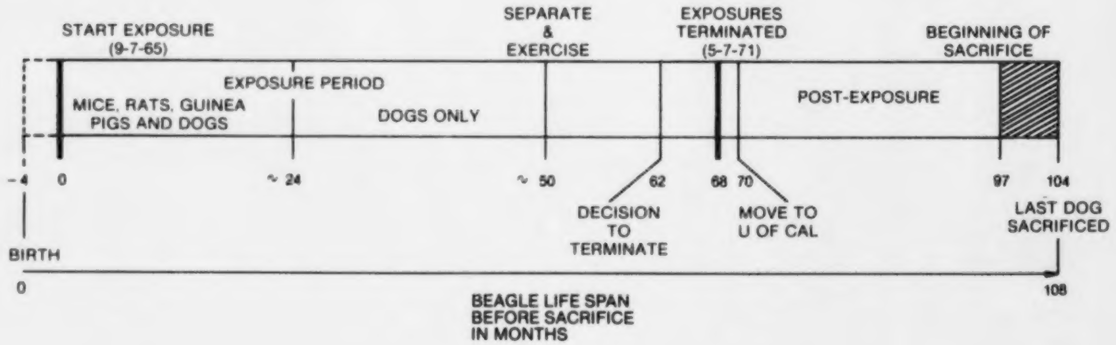

START EXPOSURE
(9-7-65)
EXPOSURE PERIOD
MICE, RATS, GUINEA PIGS AND DOGS
DOGS ONLY
SEPARATE & EXERCISE
EXPOSURES TERMINATED
(5-7-71)
POST-EXPOSURE
BEGINNING OF SACRIFICE
-4
0
~ 24
~ 50
62
68
70
97
104
DECISION TO TERMINATE
MOVE TO U OF CAL
LAST DOG SACRIFICED
BIRTH
0
108
BEAGLE LIFE SPAN BEFORE SACRIFICE IN MONTHS

Figure 2
Study time line.

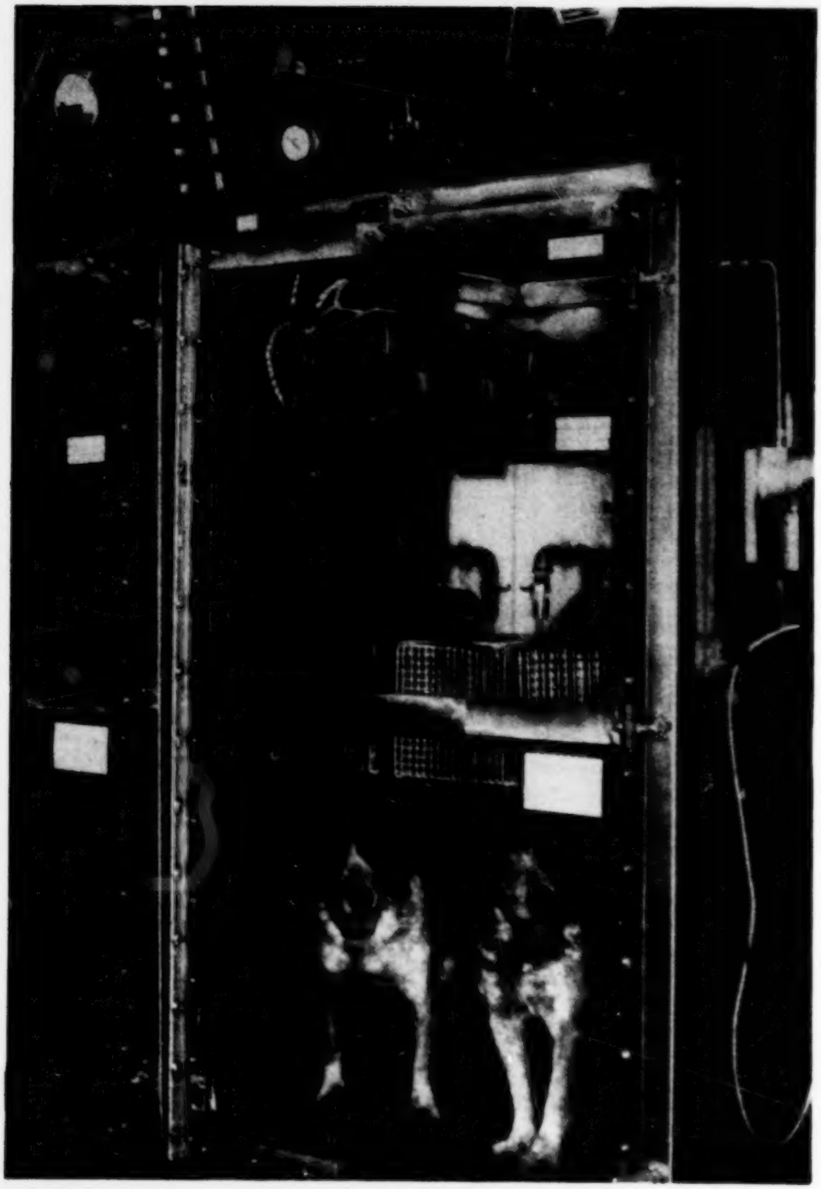


Figure 3
Inhalation chamber with placement of experimental animals. (Two other dogs are located in the rear of the chamber.)

Standard deviations and fractiles of frequency distributions were also calculated but are not shown. (For details see Chapter 2.)

Experimental Design

The investigation was designed in a spirit of complete cooperation between the statisticians and the other senior scientists who were conducting the biological studies. The statistics staff was asked to participate in the design of the study, and even though some compromises had to be made based on engineering design realities, the study was based on a sound statistical experimental design. The aspect of the experimental design given the greatest attention was the control of different types of experimental error. The first among the basic types of errors considered was *inter-animal biological variability*, i.e., the failure of different animals to exhibit exactly the same response under the same conditions. We know, for example, that this error is large for white blood cell counts when compared to red blood cell counts. The coefficient of variation for inter-animal variation was as high as 35% for white blood cell counts, which means that there could be 70% variation about the mean (at the 95% probability level). The variation was only about one-half as great for red blood cell counts. The second type of error considered was *intra-animal sampling variation*. For many types of

Table 2. Composition of Exposure Atmospheres and Abbreviations Used

Abbreviation	Atmosphere
CA	Control air
R	Non-irradiated auto exhaust
I	Irradiated auto exhaust
SO_x	0.5 ppm SO_2 + 100 $\mu g/m^3$ H_2SO_4 mist in control air
R + SO_x	Non-irradiated auto exhaust + 0.5 ppm SO_2 + 100 $\mu g/m^3$ H_2SO_4
I + SO_x	Irradiated auto exhaust + 0.5 ppm SO_2 + 100 $\mu g/m^3$ H_2SO_4
$NO_L + NO_{2H}$	0.2 ppm nitric oxide + 0.5-1.0 ppm nitrogen dioxide
$NO_H + NO_{2L}$	1.5-2.0 ppm nitric oxide + 0.2 ppm nitrogen dioxide

Table 3. Estimated Exposure Concentrations (0800 to 2400 hr daily for 68 months)[a]

Component	R	I	SO_x	$NO_L + NO_{2H}$	$NO_H + NO_{2L}$
CO (ppm)	100	100	—	—	—
HC as CH_4 (ppm)	24-30	24-30	—	—	—
NO_2 (ppm)	0.1	0.5-1.0	—	0.5-1.0	0.2
NO (ppm)	1.5-2.0	0.1	—	0.2	1.5-2.0
O_x as O_3 (ppm)	—	0.2-0.4	—	—	—
SO_x (ppm)	—	—	0.5	—	—
H_2SO_4 ($\mu g/m^3$)	—	—	100	—	—

[a] The actual measured concentrations are given in Table 4 of Chapter 2.

Table 4. Comparison of Pollutant Levels in Chronic Dog Study with Ambient Atmospheric Concentrations in Four Cities — 1966-1971

		1966[a]	1967[b]			1968[b]			1969[a]			1970[b]			1971[a]			
City	Atm. (ppm)	Max. 24-hr. Means	Max. 24-hr. Means	Ann. Arith. Means	Max. Ann. Value	Max. 24-hr. Means	Ann. Arith. Means	Max. Ann. Value	Max. 24-hr. Means	Ann. Arith. Means	Max. Ann. Value	Max. 24-hr. Means	Ann. Arith. Means	Max. Ann. Value	Max. 24-hr. Means	Ann. Arith. Means	Max. Ann. Value	Experimental Exposure Levels In Dogs
Chicago	CO	32.5	22.9	—	—	16.3	6.2	40.0	23.5	—	41.0	14.0	6.4	35.0	15.7	—	29.0	100
	NO	0.34	0.189	0.072	—	0.213	0.072	0.61	—	0.08	1.6	—	0.09	1.8	—	—	1.8	1.5-2.0
	SO_2	0.55	0.645	0.125	—	0.437	0.117	—	1.61	0.07	1.6	0.23	0.04	0.50	0.23	—	0.68	0.50
	NO_2	0.15	0.114	0.050	—	0.096	0.048	0.17	0.13	0.05	0.17	0.13	0.058	0.20	0.42	0.06	0.57	0.5-1.0
	HC	5.7	5.18	3.00	—	5.16	2.9	12.3	6.3	2.7	8.1	3.7	2.0	7.4	4.5	2.4	8.1	24-30
	Oxid.	0.08	0.077	0.029	—	0.113	0.024	0.18	—	—	0.07	—	—	0.16	0.06	0.02	0.17	0.2-0.4
Cincinnati	CO	9.3	13.0	5.6	—	32.4	5.6	42.0	10.9	—	24.0	7.2	—	21.0	9.7	—	24.0	100
	NO	0.37	0.358	0.032	—	0.339	0.064	1.02	—	—	0.71	—	—	0.80	—	—	0.62	1.5-2.0
	SO_2	0.13	0.330	0.021	—	0.097	0.017	—	0.07	—	0.29	0.09	—	0.50	0.05	—	0.24	0.50
	NO_2	0.09	0.058	0.028	—	0.100	0.031	0.056	0.07	—	0.22	0.10	—	0.24	0.08	—	0.17	0.5-1.0
	HC	(4.7)[d]	4.61	2.51	—				5.3	2.7	11.2	3.8	—	7.4	5.3	—	8.6	24-30
	Oxid.	0.6	0.089	0.031	—	0.054	0.022	0.14	0.08	—	0.16	0.03	—	0.08	0.07	—	0.16	0.2-0.4
Washington, D.C	CO	15.4	14.6	4.9	—	13.8	3.3	—	10.9	—	25.0	13.8	3.5	25.0	9.9	3.5	46.0	100
	NO	0.41	0.359	0.047	—	0.305	0.035	—	—	—	1.2	—	0.05	1.2	—	0.04	0.63	1.5-2.0
	SO_2	0.25	0.132	0.048	—	0.180	0.036	—	0.10	—	0.17	0.08	0.02	0.21	0.08	0.03	0.20	0.50
	NO_2	0.07	0.088	0.043	—	0.082	0.047	—	0.09	—	0.24	0.15	0.052	0.30	0.12	0.038	0.74	0.5-1.0
	HC	6.4	—	—	—	5.72	2.23	—	3.5	2.0	9.4	5.2	1.8	11.6	4.2	1.9	12.0	24-30
	Oxid.	0.10	0.080	0.025	—	0.095	0.027	—	0.05	—	0.10	0.06	—	0.16	0.07	0.025	0.24	0.2-0.4
Los Angeles	CO								20.2	6.5	39.0	17.2	6.0	45.0	13.6	5.4	36.0	100
	NO								—	0.098	1.2	—	0.11	1.2	—	0.11	1.1	1.5-2.0
	SO_2								0.05	0.02	0.09	0.06	0.02	0.20	0.04	0.02	0.11	0.50
	NO_2								0.24	0.065	0.80	0.22	0.074	0.30	0.28	0.092	0.74	0.5-1.0
	HC								7.0	2.9	12.0	6.8	3.2	11.6	8.4	3.6	12.0	24-30
	Oxid.								0.09	0.032	0.30	0.08	0.03	0.16	0.08	0.025	0.24	0.2-0.4

[a] NADB (AEROS, US EPA, Office of Air Quality Planning and Standards).

[b] Air Quality Data for 1967 & 1968: From NASN and contributing state and local networks. APTD-0741, 1971.

[c] Only data available.

[d] Incomplete data.

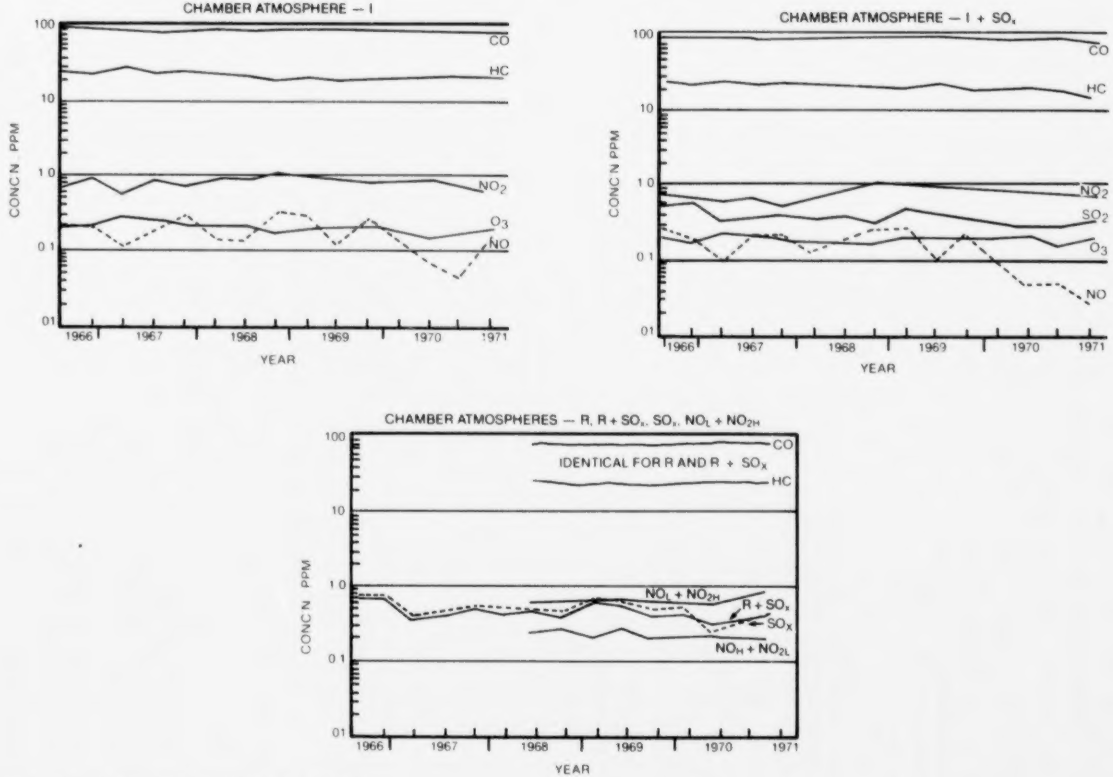


Figure 4
Five-year summary of experimental atmospheric levels.

biological measurements, intra-animal sampling variation within the same animal can be considerable. For example, one respiration may not yield exactly the same respiratory function measurement as another respiration. Such data reflect intra-animal sampling variability. An *assay error* is a failure to measure reproducibility constituents present in a particular sample in repeated analyses using the same assay technique. For example, several assays of the number of cells in a single blood sample may yield several different numerical values. The differences observed are due to assay error. Another possible error in this study was the *intra-chamber induced error*, which can be due to the movement of the animal about the chamber, to variations in the quality of the atmospheres that were ducted into the chamber over time, and to the possibly unequal pollutant distributions within the chamber space. There are degrees of variation realized between chambers receiving the same type and level of experimental atmospheres, the so-called *inter-chamber induced variation*, which may be an even greater source of error. These possible errors were controlled by physical means if at all possible, or mathematically through such devices as covariance analysis, e.g., relating an animal's post-exposure response to its pre-exposure response, or by arranging adequate controls and test extra space groups within homogeneous sets of conditions, i.e., within "blocks." In Table 5 the different methods for reducing the five basic types of errors are indicated. In this experiment, we decided blocking was unnecessary because we were able to eliminate most of the sources of variation that would relate to blocking (e.g., noise and light) in the exposure rooms. In addition, the animals represented a very homogeneous population. The physical steps that were taken to create a statistically valid experimental design included the purchase of dogs in two shipments from the same supplier, and assembly of the dogs as a stock colony before the study began so that groups could be chosen at random from the total pool of available animals for the different exposures. With the exceptions mentioned below, allocation of animals to specific exposures and assignments of animals to the triplicate exposure chambers for each atmosphere were made at random. Some judicious compromises of complete randomization were needed to prevent bias. In one instance there were two sisters which were separated. In another case, the random selection would have paired the largest dog with a very small animal. They were not placed together so that the small animal would not be subjugated or dominated by the larger dog. Aside from these few instances, a complete randomization was performed. One of the farsighted statistical features for the

Table 5. Methods for Reducing Errors in Animal Exposure Experiments

	Methods for reducing errors			
Basic error type	(1) Physical	(2) Mathematical	(3) Blocking	(4 & 5) Replication (with randomization)
1) Inter-animal biological variability	—	X	X	X
2) Intra-animal sampling (sample of blood)	—	—	—	X
3) Assay error	X	—	X	X
4) Intra-chamber induced variations	—	—	X	X
5) Inter-chamber induced variation	—	—	X	X

design was the use of a random order of sampling. All blood sampling, the analyses of the samples, the lung function measurements, etc., were performed in random order over the entire period of the study. The statistician furnished typewritten randomized schedules to the biologists for selecting the animals for sampling. Independent randomizations were done for each sampling period. Theoretically and practically, this was a very good system. In summary, even though an attempt was made to control the known sources of errors by other techniques mentioned earlier (e.g., blocking, mathematical corrections and differencing), still the randomization method was a useful statistical device because it controlled or prevented other unknown errors or bias and allowed them to be dealt with by probabilistic methods in statistical analyses.

As mentioned earlier, the dogs were assigned randomly to the chambers, and the treatments were applied to the chambers randomly with respect to chamber locations so that factors such as human activity, noise, light, air from the halls when the doors were opened, etc., would not confound the experiment. The triplicate exposure chambers were distributed randomly throughout the array of 28 chambers. The only restriction was that the SO_x chambers were located closer to the source of the gas, since SO_x atmosphere deteriorates rapidly in long ducts. The location of the SO_x chambers was not systematic with respect to any other factors.

Statistical analyses of the biological test data were performed using appropriate methods. Analysis of variance was used to analyze biological measurement data. "Treatment" and "chambers by treatments" were considered as sources of variation in the analysis. The sources of variation and a sample analysis of variance of red blood cell counts on 96 dogs are shown in Table 6. Expected mean squares indicate that the "chambers" mean square is the proper denominator for the F-ratio, which tests for "treatment" effects. However, in the absence of significant "chambers" effects, alternative tests were performed utilizing animal variability as the error variance.

It was believed that the results obtained on different sampling days in a given period would be statistically homogeneous, therefore day of sampling was omitted from the analysis of data. The design of the experiment included three embedded 2 x 2 factorial treatment arrangements as follows:

i) R x SO_x	Raw exhaust (2 levels)	
	CA	R
SO_x (2 levels)	SO_x	R + SO_x

ii) I x SO_x	Irradiated exhaust (2 levels)	
	CA	I
Exhaust vs. NO_x (2 levels)	SO_x	I + SO_x

iii) (Auto exhaust vs. NO_x) x Irradiation	Irradiation (2 levels)	
	R	I
SO_x (2 levels)	$NO_{1H}+NO_{2L}$	$NO_{1L}+NO_{2H}$

These factorial arrangements were exploited to improve the sensitivity of the data analysis.

Table 6. Analysis of Variance of Red Blood Cell Counts (RBC) on 96 Young Female Beagles[a]

Source of variation	Degrees of freedom	Mean square ($\times 10^{12}$)	F	Probability	EMS (Expected mean square)
Atmospheres (treatments, T)	7	4.02	4.12	0.009	$\sigma_A^2 + 2\sigma_B^2 + 8\sigma_C^2 + 24O\ (T)$
Chambers within atmospheres (C)	16	0.98	1.07	0.40	$\sigma_A^2 + 2\sigma_B^2 + 8\sigma_C^2$
Animals within chambers (biological variation, B)	72	0.91	25.06	0.000	$\sigma_A^2 + 2\sigma_B^2$
Duplicate assays (A)	96	0.03	—	—	σ_A^2

[a] Experimental Design:

8 atmospheres: 7 contaminated, 1 clean air control
3 chambers per atmosphere
4 female dogs per chamber

The 7 degrees of freedom for "treatments" were partitioned into single degree-of-freedom contrasts corresponding to main effects and interactions for the above-listed 2 x 2 factorial arrangements. The details of the statistical approaches used are referenced (9,10).

Exposure Facilities

The exhaust gases were generated by an automobile engine coupled to a dynamometer unit designed to produce exhaust according to an established driving pattern (Figure 5). Clean (CBR-filtered), conditioned air was mixed with the gases in a dilution system to produce the desired concentration. From the dilution system, the exhaust gas mixture was divided with one part flowing directly to animal exposure chambers, and the remainder flowing through irradiation chambers to other animal chambers (Figure 6). The irradiation chambers (Figure 7) were lighted by a composite of three types of fluorescent tubes designed to simulate sunlight. The facility included 28 animal exposure chambers and six irradiation chambers (see Figure 8).

To approximate the exhaust generated by an automobile in city traffic, the engine dynamometer unit was accelerated and decelerated as an automobile would be in moving traffic. In a 5-minute period, the engine cycle was the following: acceleration from a 10- to 30-mph cruise, deceleration to 15 mph, acceleration to a 30-mph cruise, and deceleration back to 10 mph. The diluted exhaust generated during a 5-minute engine cycle had a variation in the CO content of 50 to 180 parts per million (ppm) with an integrated average of 100 ppm.

Dilution System

The engine-dynamometer unit generated an average CO concentration of approximately 57,000 ppm or 5.7% by volume over the 5-minute engine cycle. After cooling in a heat exchanger and passing through a surge tank, the raw exhaust entered the dilution system where it was mixed with clean air at the throat of a venturi. The venturi throat was maintained at a negative pressure of 1 inch of water. The dilution ratio of clean air to auto exhaust gas for an atmosphere of 100 ppm carbon monoxide was 570 to 1. This required a raw-exhaust flow rate of 0.114 cubic foot per minute (cfm) to the throat of the mixing venturi.

To provide a means for adjusting the dilution ratio, a clean air line controlled by a vernier needle valve was connected to a 'T' connection in the raw-exhaust line before the mixing venturi. This needle valve was rotated by the correction servo motor on the output of the automatic control system. To measure the CO content, a sample cylinder was used to integrate the fluctuations over a 5-minute engine cycle. During the 2½-minute measurement and correction time, the average diluted gas sample was pumped by the sample cylinder piston to an infrared CO analyzer. A direct current proportional to the CO content of the gas sample from the output of the analyzer was used to energize the moving coil of a meter relay. The contacts of the meter relay were positioned at the high and low limits of the required CO concentration.

The direction of rotation of the needle valve correction servo motor was determined by whether the high or low limit contacts of the meter relay were closed. If the limits imposed on the CO concentration were not exceeded, the correction servo motor did not operate and the amount of clean air added to the raw exhaust remained constant.

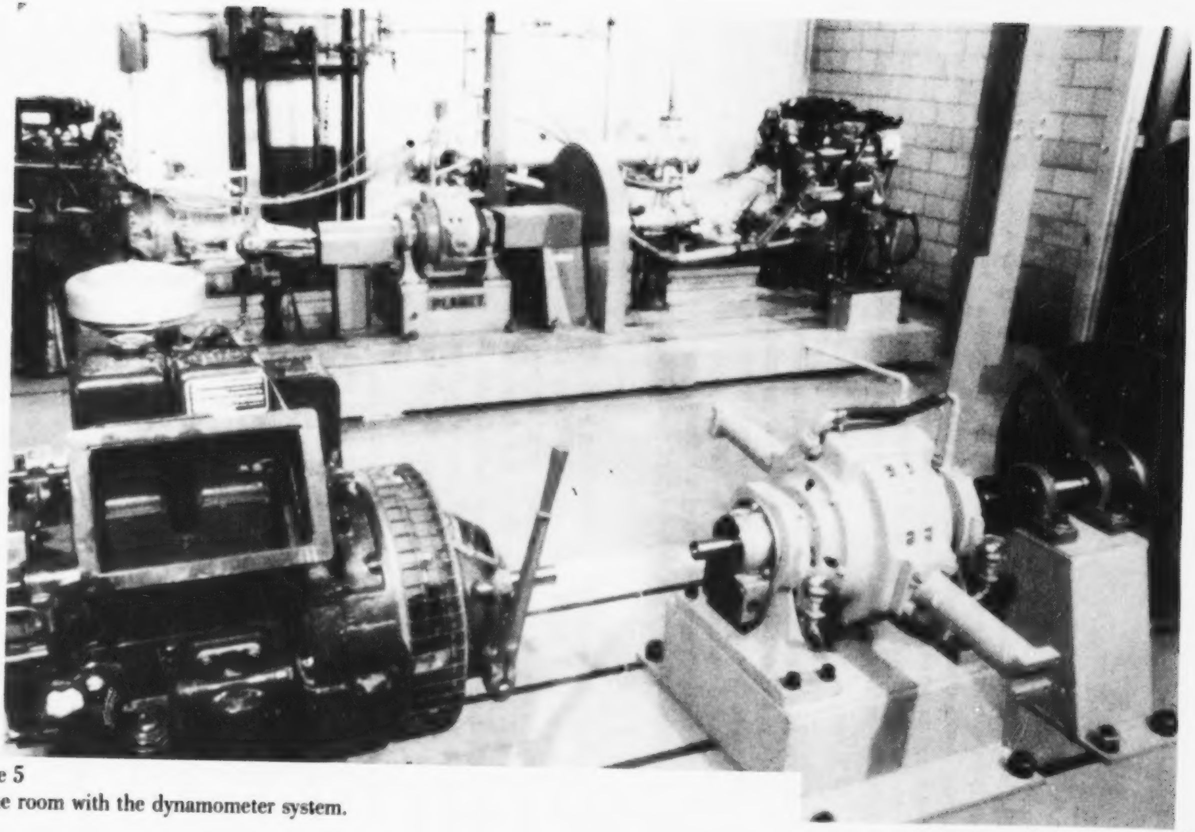


Figure 5
Engine room with the dynamometer system.

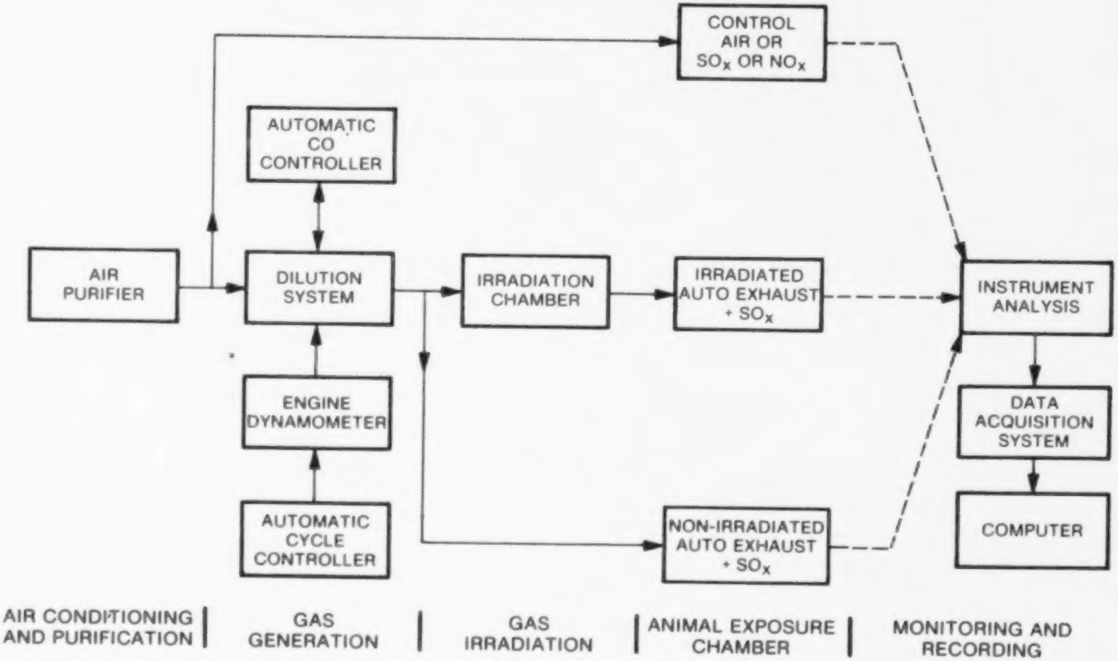

CONTROL AIR OR SO_x OR NO_x
AUTOMATIC CO CONTROLLER
AIR PURIFIER
DILUTION SYSTEM
IRRADIATION CHAMBER
IRRADIATED AUTO EXHAUST + SO_x
INSTRUMENT ANALYSIS
ENGINE DYNAMOMETER
DATA ACQUISITION SYSTEM
AUTOMATIC CYCLE CONTROLLER
NON-IRRADIATED AUTO EXHAUST + SO_x
COMPUTER
AIR CONDITIONING AND PURIFICATION
GAS GENERATION
GAS IRRADIATION
ANIMAL EXPOSURE CHAMBER
MONITORING AND RECORDING

Figure 6
Chronic auto exhaust study diagram.

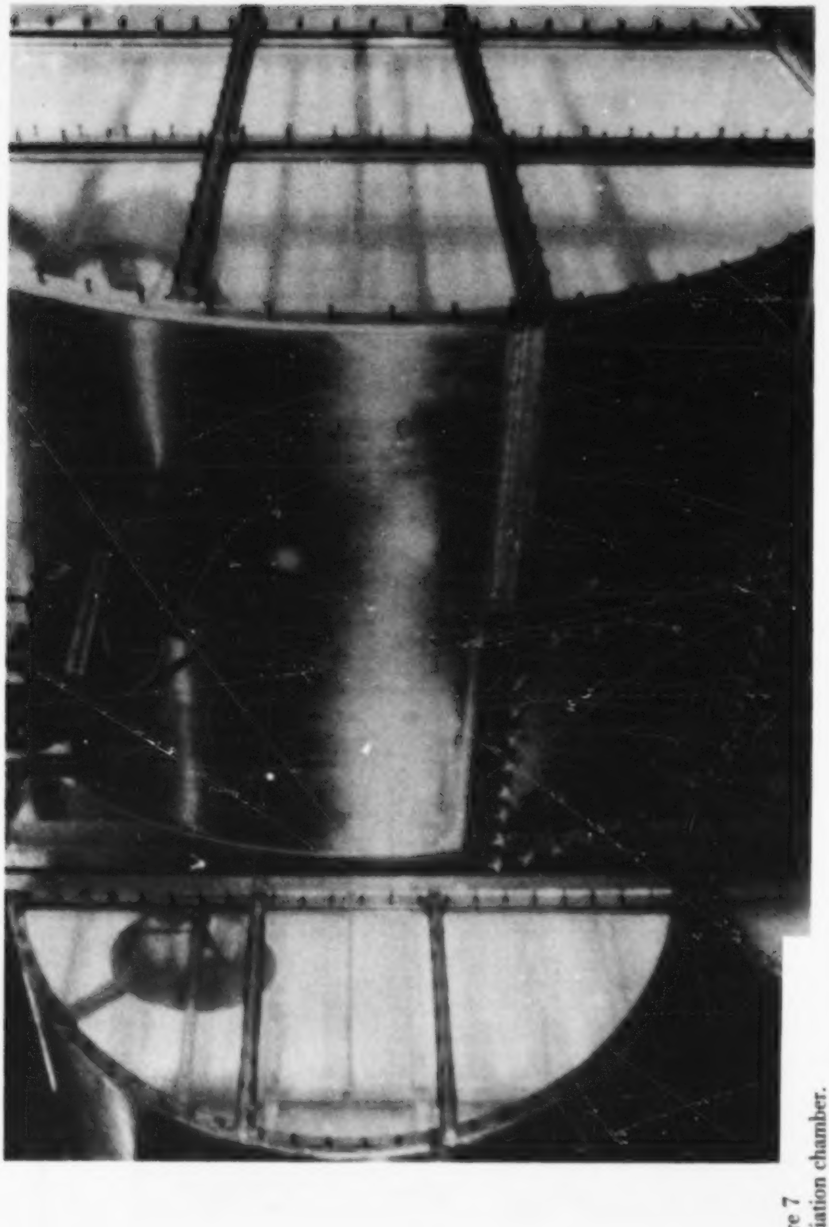


Figure 7
Irradiation chamber.

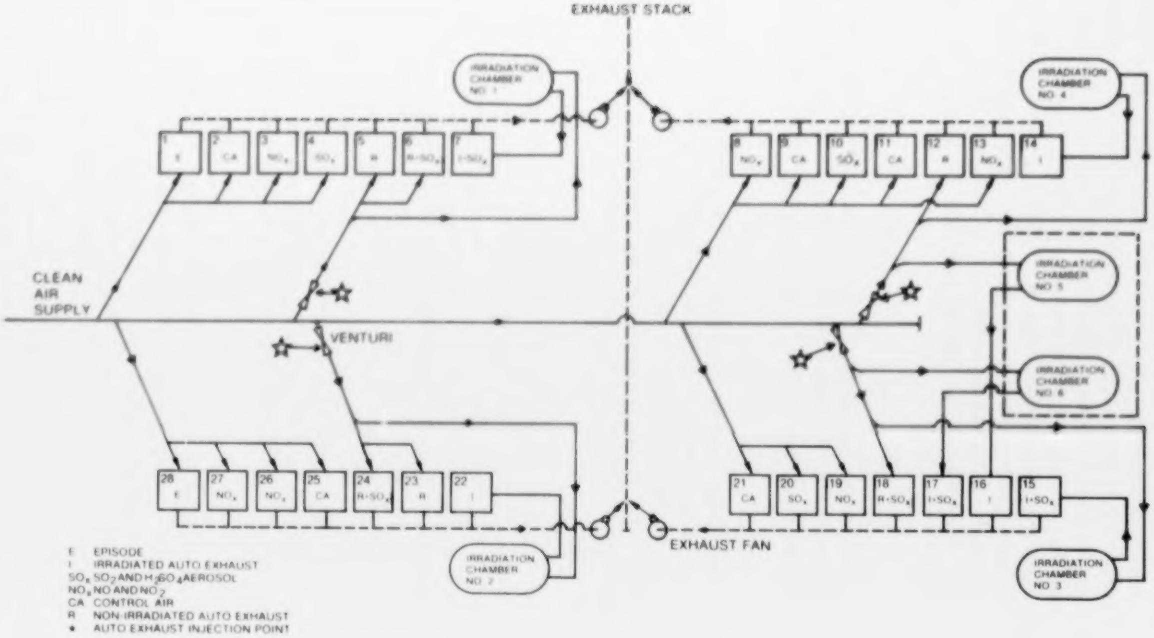

EXHAUST STACK
IRRADIATION CHAMBER NO 1
IRRADIATION CHAMBER NO 4
IRRADIATION CHAMBER NO 5
IRRADIATION CHAMBER NO 6
IRRADIATION CHAMBER NO 2
IRRADIATION CHAMBER NO 3
CLEAN AIR SUPPLY
VENTURI
EXHAUST FAN
1 E
2 CA
3 NO$_x$
4 SO$_x$
5 R
6 R+SO$_x$
7 I+SO$_x$
8 NO$_x$
9 CA
10 SO$_x$
11 CA
12 R
13 NO$_x$
14 I
15 I+SO$_x$
16 I
17 I+SO$_x$
18 R+SO$_x$
19 NO$_x$
20 SO$_x$
21 CA
22 I
23 R
24 R+SO$_x$
25 CA
26 NO$_x$
27 NO$_x$
28 E
E EPISODE
I IRRADIATED AUTO EXHAUST
SO$_x$ SO$_2$ AND H$_2$SO$_4$ AEROSOL
NO$_x$ NO AND NO$_2$
CA CONTROL AIR
R NON-IRRADIATED AUTO EXHAUST
☆ AUTO EXHAUST INJECTION POINT

Figure 8
Exposure chamber supply and exhaust flow diagram.

The system schematic diagram in Figure 9 shows only one secondary system, since all four were similar. The primary dilution occurred at point "A" where the air pressure was throttled by a vernier needle valve described above. Secondary dilution took place at the venturi, where negative throat pressure pulled the primary mixture into the duct system. Dampers affecting the system are symbolized in Figure 9. Adjustment of any dampers resulted in a change in CO concentration. The heat exchanger or surge tank "dump" dampers forced more raw exhaust into the secondary when closed. The secondary and bleed damper were operated simultaneously to maintain 33 cfm flowing in the main to the chambers. Each of the four secondary mains had three branches so that the system of 12 chambers receiving auto exhaust was balanced.

Instrumentation

The instrumentation was designed to analyze 29 gas samples (9 SO_2, 6 O_3, 6 HC, 6 CO, 1 NO, and 1 NO_2) by time-sharing the sample gases into a Flame Emission SO_2 Analyzer, a Hydrogen Flame HC Analyzer, an Infrared CO Analyzer, and a Ratio Photometer NO-NO_2 Analyzer.

Sample lines of ¼-inch Teflon from the animal exposure chambers terminated in stainless-steel solenoid-valve-operated manifolds located on the instrument table. The sample gases were routed to the appropriate gas analyzer through the manifolds. The solenoid valves were operated by selector switches on the multipoint strip chart recorders so that the gas-sample line corresponding to the point to be printed on the recorder was open to the gas measurement instrument.

The output of the SO_2 analyzer was connected to the input of a 12-print multipoint strip chart recorder. The SO_2 concentrations in nine exposure chambers were sequentially recorded on the first nine prints of the recorder. The tenth print of the recorder was used to indicate the SO_2 concentration in the exposure-chamber room and was connected to an alarm system to protect the operating personnel in case of a gas leak. The eleventh and twelfth prints were not used. The multipoint selector switch on the recorder sequentially operated the solenoid valves on the manifold connected to the input of the SO_2 analyzer. Consequently, the SO_2 analyzer received the gas sample represented by a point on the recorder at the time when that point was to be printed. The SO_2 manifold was supplied with sample gases from three exposure chambers fed with irradiated auto exhaust plus bottled SO_2 gas, three chambers fed with nonirradiated auto exhaust plus SO_2, and three chambers supplied with clean air and bottled SO_2 gas.

Eighteen points of a 24-print multipoint strip chart recorder were used to record the concentrations of O_3, CO, and HC in six animal exposure chambers fed with irradiated auto exhaust. A 19th point was used to monitor the CO concentration in the exposure-chamber room and sound the alarm if a leak developed. The multipoint selector switch on this recorder controlled the solenoid valves on the CO-HC input manifold allowing only the gas sample, represented by the corresponding point on the recorder, to flow into the CO and HC gas analyzers. Another section of the multipoint switch connected the input of the recorder to the signal output of the six O_3 meters, the CO analyzer, and the HC analyzer in the sequential order of O_3-CO-HC for each of the six exposure chambers. The solenoid valve at the termination of the exposure-chamber sample line and the input of the CO-HC manifold was opened when the selector switch reached the O_3 position, and remained open until the HC

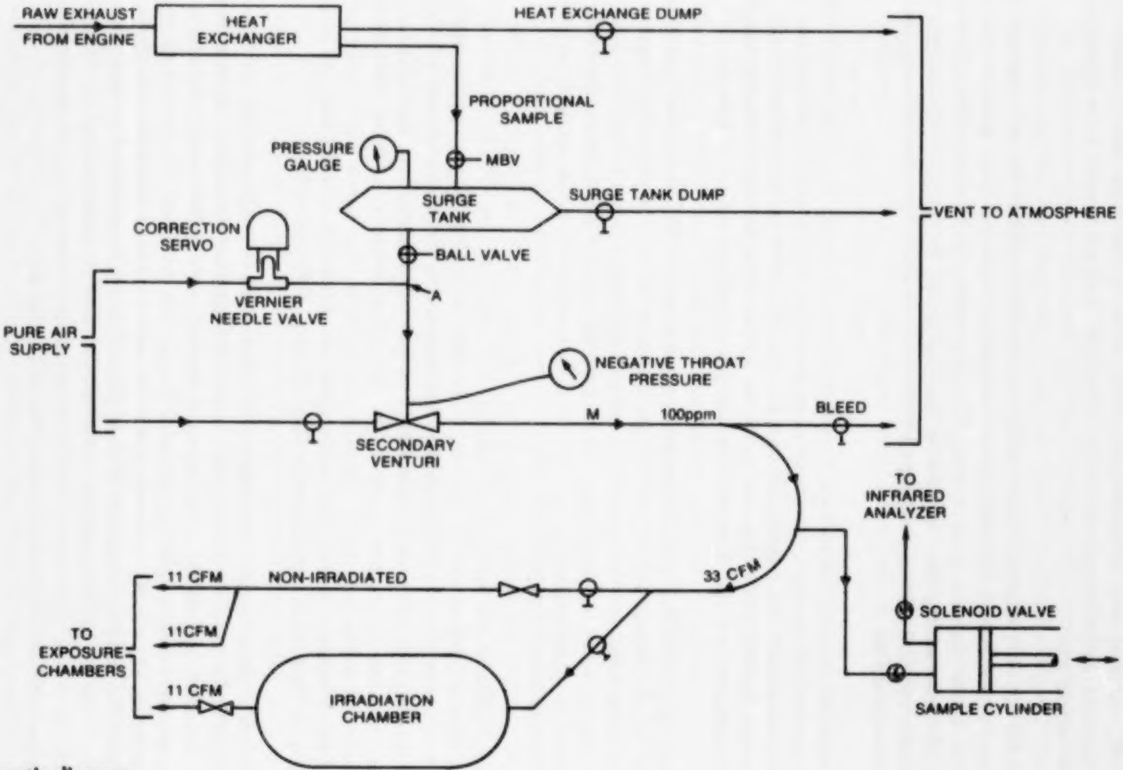

RAW EXHAUST
FROM ENGINE
HEAT EXCHANGER
HEAT EXCHANGE DUMP
PROPORTIONAL SAMPLE
PRESSURE GAUGE
MBV
SURGE TANK
SURGE TANK DUMP
VENT TO ATMOSPHERE
CORRECTION SERVO
BALL VALVE
A
VERNIER NEEDLE VALVE
PURE AIR SUPPLY
NEGATIVE THROAT PRESSURE
M
100ppm
BLEED
SECONDARY VENTURI
TO INFRARED ANALYZER
33 CFM
11 CFM
NON-IRRADIATED
11CFM
TO EXPOSURE CHAMBERS
11 CFM
IRRADIATION CHAMBER
SOLENOID VALVE
SAMPLE CYLINDER

Figure 9
System schematic diagram.

concentration for the chamber had been recorded. As a result, the solenoid valves on the CO-HC manifold were switched every three points on the strip chart recorder. This arrangement allowed enough time for the CO and HC analyzers to settle at the concentration in the sample line.

The NO and NO_2 concentrations in six animal exposure chambers fed with irradiated auto exhaust were measured by a ratio photometer analyzer connected to the input of a two-print multipoint recorder. One point recorded NO and the second point recorded NO_2. Because of the 30-minute settling time required by this instrument, the inputs to the analyzer were manually switched from one exposure chamber sample line to the other by a toggle-valve-operated manifold.

A calibration manifold was added to the system to provide a means of putting conditioned air, CO standard, HC standard, nitrogen or a bag sample into the CO and HC gas analyzers. This was accomplished with toggle-switch-operated solenoid valves for each calibration gas, and three-way solenoid valves that interrupted the output of the automatic sampling manifold. The standards used were 90 ppm CO in nitrogen and 40 ppm carbon in nitrogen.

Sample gases from four animal exposure chambers supplied with non-irradiated auto exhaust could be put through the HC and CO gas analyzers by manually operating a toggle-valve-operated manifold and the toggle-switch-operated three-way solenoid valves. The toggle valve was used to select the sample gas to be routed through the gas analyzers. The three-way solenoid valves interrupted the flow of irradiated gas samples from the manifold controlled by the multipoint strip chart selector switch.

Data Acquisition and Safety Alarm Systems

The data acquisition system provided a periodic record of 29 gas samples registered sequentially on punched paper tape. The main function of the data system was to eliminate the necessity of transferring the information on gas concentrations from the multipoint strip chart recorders for computer analysis. The data system contained a digital clock, an input scanner, an integrating digital voltmeter and a paper tape punch with parallel-to-serial coupler. A sequence control unit synchronized the data system with the three independent multipoint strip chart recorders controlling the input of the gas analyzers.

The safety alarm system was designed to indicate unsafe conditions both for personnel working in the exposure-chamber room and for the experimental animals. Because some of the pollutants fed into the animal exposure chambers were bottled gases, a warning system was necessary to alert operating personnel if a gas leak developed. The instruments used to measure the CO and SO_2 concentrations in the animal exposure chambers were also used to measure the room concentrations of these gases. This was accomplished by adding solenoid valves to the CO and SO_2 manifolds, which allowed room air to flow through the CO analyzer when the O_3-HC-CO recorder was on its nineteenth or last point, and through the SO_2 analyzer when the SO_2 recorder was on its tenth or last point.

Engine — Dynamometer Unit

The source of auto exhaust emissions was one or the other of two identical six-cylinder, 144-cubic-inch gasoline engines (see Figure 5). Each was equipped with an automatic transmission and could be coupled to opposite ends of the dynamometer unit. Both engines had the necessary fuel, ignition, and cooling systems for separate operation with a minimum of time required for switching from one engine to the other.

The dynamometer power absorption unit was an air gap, eddy current, double ended, dual rotational type that used water cooling and had a power absorption rating of 50 horsepower in a speed range of 1600 to 6000 rpm. A 300-pound flywheel was directly coupled to the dynamometer unit to provide the rotational energy to simulate the inertial mass of a moving 2500-pound vehicle. The dynamometer field current could be set at constant value or made dependent on the flywheel speed. Road speed and load conditions versus cycle time were programmed and recorded to maintain reproducibility of operation. Drive shaft speed was monitored by a built-in "Tach-Generator" and a load cell on the torque arm of the dynamometer provided load readout.

Air Purification Unit

The cleanliness, temperature, and humidity of the dilution air used in the irradiation chambers duplicated that encountered in clean urban atmospheres prior to contamination. The air purifying system consisted of a pressure blower, filters, heat transfer coils, and a humidifier combined into a single unit, together with a separate refrigeration compressor and cooling tower. This portion of the facility supplied approximately 500 cfm of air at a pressure of an 8-inch water column above atmospheric, measured at the outlet of the humidifier section. The pneumatic controls were completely automatic once outlet temperature and relative humidity had been set on the appropriate controllers.

The 7.5-ton capacity refrigeration compressor was a water-cooled type with an unloading control. A forced-draft indoor cooling tower supplied recirculating cooling water to the condenser coils. The sensing element for the unloader, located in the exit side of the cooling section, actuated a thermostat-operated switch that controlled the compressor unloader and maintained the cooling coil at the required operating temperature. Saturated air at 40° dry bulb leaving the coil was then brought to the desired final conditions by the temperature and humidity controls.

Hydrocarbons, organic and inorganic gases, and solid particulates, were reduced to acceptable levels by a filtering system. A prefilter 2 inches thick on the intake of the blower was followed by four absolute filter elements to remove particles down to 0.3 micron diameter, and four activated charcoal filters to remove odors and gases. The entire air distribution system ahead of the exposure chambers was constructed of stainless steel wherever the air was in contact with a metal surface.

Irradiation Chambers

The general dimensions of each of the six irradiation chambers were 23.5 feet long by 47 inches wide by 8 feet high and each had a volume of approximately 683 cubic feet. No metals other than aluminum and stainless steel were employed in any location that came into contact with the atmosphere of the chamber. The sides were of a framework of aluminum structural members. The plastic films that formed windows to pass the irradiating energy were clamped and sealed by means of pressure screws and gasketed channels. The top, bottom, and ends of the chamber were formed of ¼-inch thick aluminum plate welded inside and outside at all seams to prevent leakage. A sliding manhole door secured in place by screw clamps was provided for access to the interior of the chamber (see Figure 7).

Sources external to the chamber supplied intense ultraviolet radiation, which passed through windows of "Teflon" FEP, type "c" fluorocarbon film, of 5 ml thickness (500 gauge). Each irradiation chamber had four banks of 74 flourescent tubes mounted in four external cavity reflectors. The 96-inch tubes were operated from ballasts remotely mounted at the ends of the chamber room and both high and low voltage leads were brought into the panels. The lighting panels were supported on trolley tracks for rolling back from the chamber side to facilitate servicing light tubes and changing window films. Three types of tubes, 16 blue light, 46 black light, and 12 sunlamps comprised the 74 tubes in each lighting panel.

The blue fluorescent lamps radiated energy in a band extending from 3000 to 6000 angstroms with approximately half the total energy radiated in a 4000- to 5000-angstrom band. A spectral peak was found at 4400 angstroms. The black fluorescent lamps radiated most of their energy in a 3000-to-5000 band, peaking sharply in the 3500-angstrom region. The sunlamps radiated ultraviolet energy in the 2800- to 3500-angstrom band. One-half of this energy, however, was radiated in a band from 3000 to 3250 angstroms, with a sharp energy peak at 3100 angstroms.

The switching was so arranged that circuit breakers on the control panel operated one-fourth of the lamps on each of the four lighting panels to provide even distribution of reduced intensities.

To maintain the lamps at their most efficient operating temperature, a system to remove the generated heat from the lighting panels was provided. This consisted of an exhaust fan taking air from the shallow plenum chambers mounted on the outside of the panel reflectors and connected to the exhaustor through duct-work on top of the chamber. Room air was drawn through the space around the four sides of the panel, through a row of 5/32-inch holes in the reflector immediately behind each lamp, and into the exhaust system. Quick-disconnect slip joints were used to connect the duct to the mating duct on the panel, with a canvas connection provided to facilitate alignment. The reflectors were painted with an indoor flat white paint containing a high magnesium oxide content as an extender in combination with titanium oxide for good reflection of ultraviolet radiation.

Animal Exposure Chamber Construction

The exposure chambers (Figures 10 and 11), constructed of 14-gauge type 304 stainless steel, were 9 feet 10 inches high, 3 feet wide and 3 feet deep. Formed funnels, top to bottom, had an angular slope of approximately 53°. A volume of 44 cubic feet, or 1,245 liters, between the supply and exhaust funnels was available for animal exposure. At the bottom of the chamber were four uncaged beagles. The access door, 54 inches high and 32 inches wide, was made of 3/8-inch plate glass set in a neoprene gasket and framed with stainless steel. The door had a full-length piano hinge and five pressure clamps, permitting pressure adjustment to seal the door tight against its neoprene gasket. The clamps were positioned at each corner opposite the hinged side and midway between the corners, both horizontally and vertically. In the rear of the chamber, a window of 3/8-inch plate glass 18 inches wide permitted observation from both sides. The lower formed-funnel section was supported in a structural-angle-iron frame. Adjusting bolts in the floor plates of the legs were provided for leveling the chamber.

Cage rack support pins were welded in the four corners of the chamber at six levels about 10 inches apart. The removable cage racks were formed of ¼-inch diameter stainless steel rods

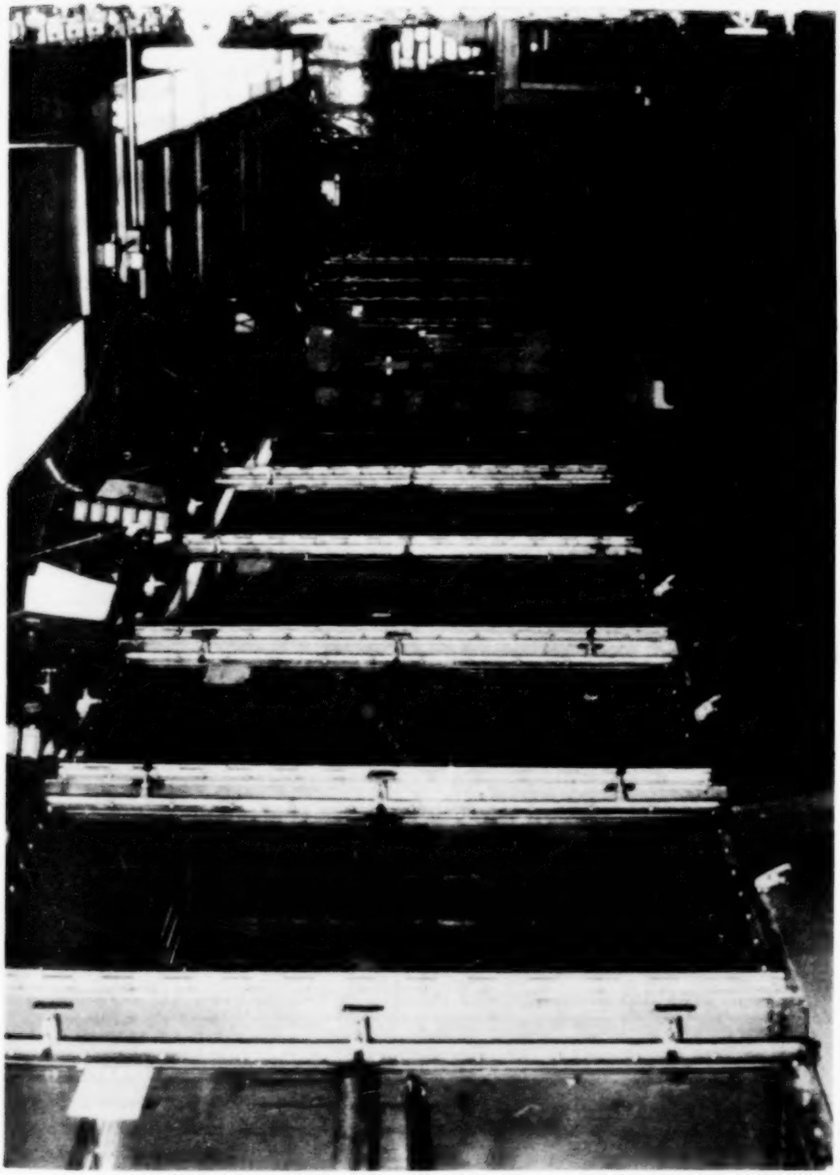


Figure 10
Animal exposure chambers.

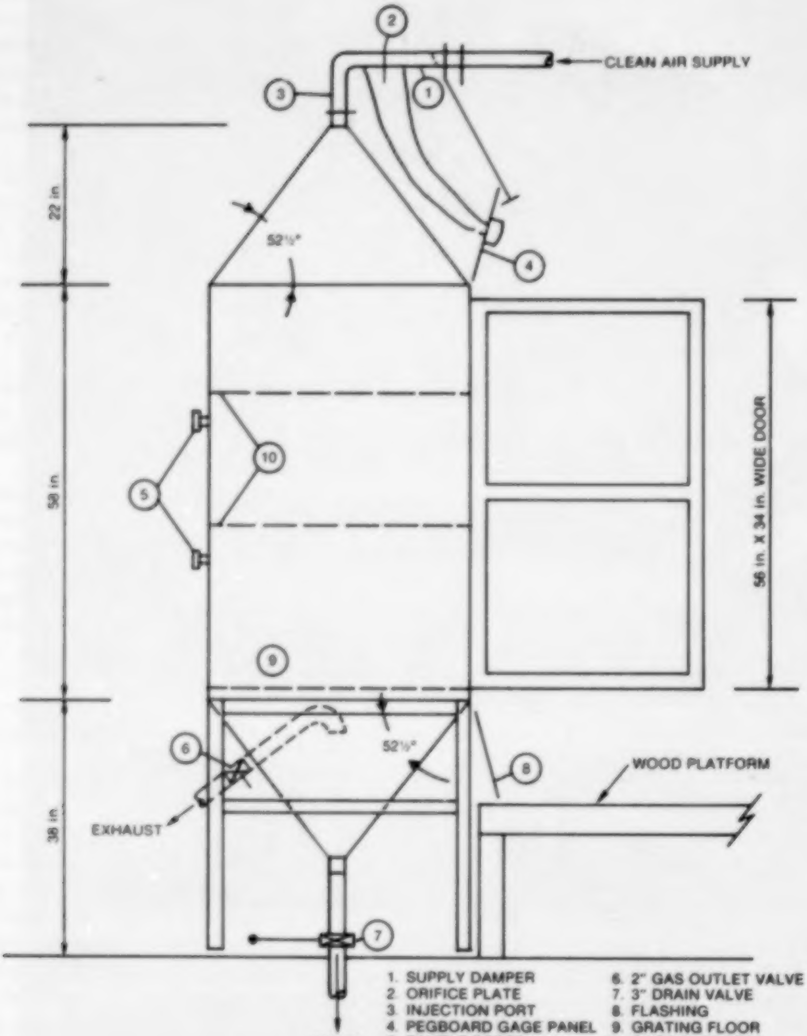

2
CLEAN AIR SUPPLY
3
1
22 in.
52½°
4
58 in.
10
5
56 in. X 34 in. WIDE DOOR
9
6
52½°
8
WOOD PLATFORM
EXHAUST
38 in.
7
WASTE
1. SUPPLY DAMPER
2. ORIFICE PLATE
3. INJECTION PORT
4. PEGBOARD GAGE PANEL
5. SAMPLE PORTS
6. 2" GAS OUTLET VALVE
7. 3" DRAIN VALVE
8. FLASHING
9. GRATING FLOOR
10. CAGE SUPPORTS

Figure 11
Animal exposure chamber — Schematic diagram.

with eyelets in the corners to engage the support pins. Cages for rats or guinea pigs hung from the racks and could slide in and out of the chamber on light-gauge stainless-steel angle spot-welded to the sides of the cages. The floor of the chamber was 12-gauge stainless steel 11/16 x 1-25/32 inches expanded metal supported on a cage rack. Each chamber had six cage racks and two expanded metal gratings. This construction allowed flexibility in chamber loading and permitted exchange of racks when the animals were serviced.

Four ½-inch-diameter iron pipe size (ips) stainless steel couplings were welded through the wall at the rear of each exposure chamber. Through one of these ports, atmosphere samples at the breathing zone of the animals were taken continuously with a stainless steel tube extending 18 inches inside the chamber wall. The gas was drawn through Teflon tubing to the centrally located gas analyzing instruments.

The negative static pressure of 0.3 inch of water in the chamber was measured with a magnehelic gauge connected by a tee fitting to the second coupling port. The third port contained a well-type dial-face thermometer for observing the temperature inside the chamber. The fourth opening was used to provide fresh drinking water to the dogs on the bottom level of the chamber by means of an automatic watering device (see Figure 11).

Airflow, Cooling System and Miscellaneous Provisions

Airflow to each exposure chamber receiving irradiated or raw auto exhaust was metered by a Venturi type flowmeter in the supply duct. In the clean air supply duct to the other chambers, orifice-type flowmeters were used. The pressure taps of each flowmeter were connected to a magnehelic pressure gauge on the panel board above each chamber. An airflow calibration curve was drawn for each flowmeter, and the gauge was marked with a red arrow for the proper flow. The flow through these meters could be varied but was set for 11 cubic feet per minute of 15 air changes per hour through each chamber. Rates of airflow and static pressure in the chamber were controlled by means of a butterfly damper located in the supply duct and a globe valve in the exhaust duct. The damper in the supply duct was manually controlled at each chamber through mechanical linkages that permitted fine adjustment. A negative static pressure of 0.3 inch of water was maintained in the operating chambers by a globe valve in the exhaust pipe. Air was exhausted through a 2-inch elbow turned downward and located beneath the floor in the center of the chamber. The elbow was connected to the exhaust header by a stainless steel pipe welded through the side of the lower funnel.

In addition to controlling the cleanliness, temperature, and humidity of the dilution air supply described earlier, the temperature rise due to the irradiation chamber lights had to be overcome. The 2-inch diameter auto exhaust lines from the six irradiation chambers to their respective six animal exposure chambers were wrapped with 1/4-inch refrigeration copper tubing for a distance of 5 feet, just before the entrance to the exposure chamber. Freon from the refrigeration compressor for the dilution air supply was circulated through a main header to each of the six locations requiring additional cooling. Solenoid valves were provided at the branches, and there was a main switch to cut off the cooling when desired. The insulated lines could lower the temperature inside the animal exposure chamber from 80 to 75°F when in operation. A seven-day circular-chart recording instrument for continuously monitoring temperature and humidity of the air used for dilution was installed to check on the performance of the air conditioning equipment.

For each irradiation chamber, there was a magnehelic gauge with a differential pressure switch preset to actuate on a 1-inch water column decreasing positive pressure. A central alarm bell was used to indicate failure of any of the Teflon fluorethylene propylene co-polymer windows in the six irradiation chambers or any other irregularity in pressure. A series of pilot lights indicated if a chamber was operating below normal, and individual switches were provided to deactivate the alarm for low pressure in each chamber. When the irradiation chamber pressure returned to normal, the alarm bell would ring again until the switch had been returned to the normal position.

One leg of the tee-fitting connected to an exposure chamber access port was provided with a magnehelic gauge. The other leg was provided with a differential pressure switch preset to actuate a red pilot light on the instrument panel above the chamber door on a decreasing negative pressure of 0.1-inch water column. The light would indicate to the operator a malfunction, such as an improperly sealed chamber door or a clogged globe valve in the chamber exhaust line. For the latter, a 2-inch tee with a plug in one leg served as a cleanout between the chamber and the common exhaust header for a row of seven chambers.

Precautions to insure a safe working environment for laboratory personnel included a 3,500 cfm exhaust fan in a room window. An abnormal concentration of CO, SO_2, or NO_2 in the exposure room from a leaking bottle gas cylinder or any ruptured lines would automatically start the fan. A pilot light on the instrument panel would light, and an alarm would ring by means of a wiring circuit connected with the chart recorders of the instrumentation that monitored the room atmosphere as well as the exposure chambers. The window exhaust fan also served as a backup system in case of failure of the exposure-room air conditioning system, which was a separate unit. Corridor air or outdoor air could be drawn through the room to provide ventilation until the air conditioning unit was repaired.

A major consideration from the operator's point of view was keeping the chambers clean. Around the perimeter of the chamber and directly beneath the stainless steel expanded metal rack, the bottom level for animal exposure, was a 1/2-inch ips spray ring. Water from four wide-angle nozzles flushed the excreta on the bottom cone to the drain. The water was regulated to 102°F to prevent steaming inside and to the rear of the individual chambers. A 3-inch quick-opening lever-operated gate valve located at the bottom of the chamber was opened when the water spray ring was turned on. The drain valves from a row of chambers were connected to a common line leading to the city sewer. Another water line discharged into the sewer where the excreta entered to provide a flushing action and keep the sewer line clean.

When the entire chamber, from top to bottom, required cleaning, the dogs were removed to a mobile cart and the interior of the chamber was hosed down. A portable unit with a hose attached to the hot water line was used where detergent chemical action was required to cut any deposit buildup. The unit had a 1½-quart refillable plastic container in which a mild acid could be used. An injector containing an adjustable valve regulated the volume of cleaning solution for best efficiency. Once the surface was completely clean, regular washing with the high-pressure water hose nozzle would keep it clean for about one week.

References

1. Stara, J.F. *et al.* 1974. Toxicology of atmospheric pollutants resulting from fuel additives and emissions associated with the use of automotive catalytic converters. Pp. 751-779 *in* Vol. II, Recent advances in the assessment of the health effects of pollution, International Symposium Proceedings (Paris, June 1974).
2. Hueter, F.G. *et al.* 1966. Biological effects of atmospheres contaminated by auto exhaust. Arch. Environ. Health 12: 553-560.
3. Stara, J.F. 1970. Technical review committee on the chronic dog study. Report to NAPCA, DHEW.
4. Stara, J.F. 1970. Chronic toxicity of auto exhaust and other air pollutants in beagles. Report to the Director, DHER, NAPCA, DHEW (September 1, 1970).
5. Stara, J.F. 1970. Further comments related to the termination of the chronic auto exhaust study in beagles. Memorandum to Director, DHER, NAPCA, DHEW (September 11, 1970).
6. Stara, J.F. 1969. Chronic toxicity of auto exhaust and other air pollutants in rodents and beagle dogs. Revised Study Protocol (November 7, 1969).
7. NABD (AEROS, U.S. EPA, Office of Air Quality, Planning and Standards).
8. Air Quality Data for 1967 and 1968. 1971. NASN and contributing state and local networks, APTD-0741.
9. Cochran, W.G., and G.M. Cox. 1957. Experimental designs, 2nd ed. John Wiley and Sons, Inc., New York.
10. Snedecor, G.W., and W.G. Cochran. 1967. Statistical methods, 6th ed. The Iowa State University Press, Ames, Iowa.
11. Korth, M.W. 1963. Dynamic irradiation chamber tests of automotive exhaust. Environmental Health Series, Air Pollution. U.S. DHEW, PHS.
12. Hinners, R.G. *et al.* 1968. Animal inhalation exposure chambers. Arch. Environ. Health 16: 194-206.
13. Stara, J.F. 1972. Summary of the data from the chronic auto exhaust study on the beagle. Internal Report No. 2 to Division of Health Effects, NAPCA, DHEW.
14. Stara, J.F. 1973. Chronic effects of auto exhaust, simulated smog and other pollutants in beagles. Internal Report No. 3 to Division of Processes and Effects, Office of Research and Monitoring, EPA.
15. Stara, J.F. *et al.* 1972. Chronic effects of auto exhaust and other atmospheric pollutants in female beagles. ETRL Annual Report for 1972, NERC, EPA.
16. Stara, J.F. *et al.* 1976. Long-term effects of auto exhaust and related pollutants in beagles. I. A study overview and pulmonary function effects. Proceedings of 172nd ACS Symposium, San Francisco, California, Section on Environment, ABS. No. 38.

Asilomar Conference Discussion

Stara: The animals were exposed, almost to the day, for 68 months. The other 2 months were spent performing the pulmonary function and other tests, and moving the dogs to Davis at 70 months. During this time the beagles were in the chambers but not exposed to the auto exhaust and to the other atmospheres.

Albert: Would you explain what the $NO_L + NO_{2H}$ and $NO_H + NO_{2L}$ are?

Stara: The concentrations are 0.2 of NO and 0.5 to 1 ppm of NO_2 in one instance, 1.5 to 2 ppm of NO and 0.2 ppm of NO_2 in the other instance.

Albert: These were based on the ratio of NO/NO_2 in raw and irradiated atmospheres, so this was pulling out NO_x as a pollutant and looking at it individually.

Stara: Exactly. These were the concentrations that appear in Table 3. The time line shows the series of tests that were done in Cincinnati. They are indicated with an asterisk. The remainder of the tests were done in California. About February 1969, we have separated the dogs in chambers. There were other miscellaneous tests performed. For example, Dr. Lee has done some sulfur determinations, and Dr. Johnson has studied the evoked potential (EEG) response. There was also some clinical chemistry data available. Some immune response testing was performed with rabies antigen at CDC.

Hueter: We weren't interested in the driving system. We were interested in the concentration of pollutants in the atmosphere. We ran the engine any way we had to in order to get those concentrations. The carbon monoxide level actually was the pollutant index controlling the exposure levels.

Albert: How did you make the sulfuric acid?

Stara: The sulfuric acid was produced using fuming sulfuric acid, and then evaporating it into the atmosphere of 55% humidity where it became reconstituted into sulfuric acid mist, with an average particle size of 6 microns.

Question: At what relative humidity?

Stara: About 55%. We used 28 of the inhalation chambers. Ken Busch has selected at random chambers for the various treatments according to Table 1.

Comment: How many chambers for each atmosphere?

Stara: Three chambers for each of the exposure atmospheres, five chambers for the control, for a total of 26 chambers. Two of them were used for other purposes. Table 1 is a review figure for the individual atmospheres and how many dogs were alive in which atmosphere and which chamber. Figure 11 shows a diagram of one of the chambers. The exhaust and the clean air supply was brought up from the top, and on the bottom the gaseous exhaust and the liquid wastes were eliminated. This chamber is a useful chamber for inhalation studies, but it would serve better for smaller animals like rodents, because they could be separated at three levels. A 3 ft. x 3 ft. area is just too small for four beagles. A review of the maintained gaseous concentrations is presented in Table 3.

Busch: The NO is twice as high by itself as it was in the irradiated, but it's low. It was meant to match, but it's a little bit high, 0.2 instead of 0.1.

Stara: Yes. The NO, NO_2, SO_2, ozone were supposed to be, as Dr. Hueter said, at approximately the levels found in raw and irradiated auto exhaust, and that's why they were selected.

Comment: They're nominal there. They're not actual concentrations.

Question: But it didn't work that way, did it? When you put that much SO_2 in with the irradiated, you convert a lot of the sulfuric acid. We found out after a while that we had something like 300 or 400 mg/m^3. We were killing some guinea pigs and then we had to reduce the SO_2 going in to get back to 100. Wasn't that true, Mr. Malanchuk?

Malanchuk: That's true. Again, as mentioned previously, the SO_2 is a nominal value that was aimed at, and we adjusted the supplementary supply to try to maintain the exposure chamber concentration at that level. And sometimes it would go up pretty high. That is, we wanted to maintain the SO_2 overall concentration, with sulfuric acid really. Apparently SO_2 was getting converted into sulfuric acid and we had to reduce the SO_3 flow from the supplementary fuming sulfuric acid supply to try to maintain the concentration at the nominal level.

Stara: As to the dogs, we had the four dogs at the low level of the chamber. Female beagles at this age, 4 or 5 years old, in a small enclosure become rather irritable, perhaps also because they were not mated. The animals were separated into pairs, after the leader problem was solved. They were fed during the 8-hour rest period; they had water *ad libitum*. In early 1970, we began to exercise the dogs during the 8-hour period . . . and most of the dermatitis cases and other cases were corrected. One of the points that I want to make in conclusion is that we were fortunate that we kept the animals alive after the experiment, even though they were not exposed in California, since we were able to use techniques for analysis of the tissues and pulmonary function tests that were not available at the time when the exposures were stopped. So I think it was quite fortunate that we had saved the animals and had them transferred to California where many additional and new tests were performed. I just hope that our decisions, which were of course uncertain at the time, were correct and that this extensive study will result in useful data for EPA and the scientific community.

2. EXPOSURE CHAMBER ATMOSPHERES. SAMPLING AND ANALYSIS

M. Malanchuk

Chamber Atmospheres

The exposure study facilities are described in the preceding report. Details of the system for delivering the exhaust emissions from the automobile engine to the animal exposure chambers are given in that report, as well as the preparation for monitoring the atmospheric components in the chambers.

In addition to the diluted exhaust gas, supplemental supplies of several individual gases and of sulfuric acid aerosol were introduced to certain chambers. The gases — nitric oxide (NO), nitrogen dioxide (NO_2) and sulfur dioxide (SO_2) — were metered from supply cylinders of those gases diluted in air or nitrogen. The concentrations of these cylinder gases were established at levels convenient for delivering amounts that would accurately attain the desired levels in the exposure chambers. The flow-meter readings were checked continuously and the flow rates adjusted infrequently to maintain the desired pollutant levels in the test atmospheres.

Sulfuric acid aerosol was provided by the pressure of a stream of dry air flowing over the surface of fuming sulfuric acid in a tightly enclosed Erlenmeyer flask. The sulfur trioxide (SO_3) vapor was transferred into the inlet duct at the top of the exposure chamber. The reaction of the SO_3 with the water vapor of the duct air produced the sulfuric acid needed for the chamber atmospheres. There was a total of nine chambers, each equipped with its own acid generating system. The acid supply, approximately 30 to 40 ml in a 250 ml flask, was renewed as needed to maintain a median value concentration of 100 $\mu g\ H_2SO_4/m^3$ in the chamber.

Methods of Sampling and Analysis

Automatic continuous monitoring and manual sampling checks, both regular and occasional, were carried out on the various exposure atmospheres (see Table 1). The automatic continuous monitoring focused on the irradiated (I) chamber atmospheres for measurement of carbon monoxide (CO), hydrocarbons (HC) and the nitrogen oxides (NO and NO_2) by panel-mounted instruments (see Table 2). A combination of sampling tubing network manifolds and automatically controlled solenoid valves enabled the use of one analytical instrument for each of these components in all the chambers. Ozone was continuously monitored in the I-chambers by individual detection instruments mounted on the side of each chamber, with automatic input of the electronic signal to the data acquisition system.

In addition, sulfur dioxide (SO_2) was monitored continuously in the irradiated atmosphere chambers that were supplemented with SO_2 ($I + SO_x$) as well as in the SO_2-supplied clean air (SO_x) chambers and in the SO_2-supplied chambers containing the non-irradiated exhaust emissions ($R + SO_x$). All the automatically monitored concentration values were recorded both on charts and on tape. The chart readings were used to maintain daily control over the operations of pollutant gas supply to the chambers. The tape records were used for the statistical evaluation of the data.

Table 1. Major Atmosphere Components and Measurement Strategy

Treatment	Chamber	CO	HC	NO	NO_2	O_3	SO_2	H_2SO_4
Control (clean) air (CA)	2, 9, 11, 21, 25	M	M	M	M			
Non-irradiated auto exhaust (R)	5			M	M			
	12	AM	AM	M	M			
	23			M	M			
Irradiated auto exhaust (I)	14	A	A	A	A	A		
	16	A	A	A	A	A		
	22	A	A	A	A	A		
SO_2 + H_2SO_4 (SO_x)	4						A	A
	10						A	A
	20						A	A
Non-irradiated auto exhaust + SO_2 + H_2SO_4 (R + SO_x)	6	AM	AM	M	M		A	A
	18	AM	AM	M	M		A	A
	24	AM	AM	M	M		A	A
Irradiated auto exhaust + SO_2 + H_2SO_4 (I + SO_x)	7	A	A	A	A	A	A	A
	15	A	A	A	A	A	A	A
	17	A	A	A	A	A	A	A
Nitrogen oxides (NO_L + NO_{2H})	8			M	M			
	19			M	M			
	26			M	M			
Nitrogen oxides (NO_H + NO_{2L})	3			M	M			
	13			M	M			
	27			M	M			

Key: A = Sampled and recorded automatically, continuously
AM = Sampled regularly and recorded manually
M = Sampled variably and recorded manually

Table 2. Pollutants Measured Automatically and Continuously

Component	Chamber type(s)	Principle of measurement[a]
Carbon monoxide (CO)	I, I + SO_x	Non-dispersive infra-red, NDIR (U-108)
Hydrocarbons (HC)	I, I + SO_x	Flame ionization detection, FID (U-55)
Nitrogen oxides (NO_x):		
Nitric oxide (NO)	I, I + SO_x	Colorimetry (U-97)
Nitrogen dioxide (NO_2)	I, I + SO_x	Colorimetry (U-97)
Ozone (O_3)	I, I + SO_x	Colorimetry (U-43)
Sulfur dioxide (SO_2)	I + SO_x	Flame photometric detection, FPD (U-133)
Sulfuric acid (H_2SO_4)	I + SO_x	Ion Conductivity (U-20)

[a] page no. of instrument description in *Air Sampling Instruments*, 4th ed. (1972), published by ACGIH, Cincinnati, Ohio, is provided in parentheses.

Analysis for peroxyacyl nitrates (PAN) in chamber atmospheres was fruitless. Measureable concentrations of PAN were not detected in any of the exposure chambers, nor in several of the irradiation chambers themselves.

Twice-a-day measurements were made on the non-irradiated atmospheres (one R chamber and all three R + SO_x chambers) for CO, HC, and NO_x (NO and NO_2). The manifold plus solenoid valve system was used to "cut in" the line samples to the same panel-mounted instruments used for the I atmospheres. The readings were entered manually on a data sheet for statistical processing. All the nitrogen oxide values were obtained by individually collected bubbler samples treated for colorimetric measurement (1). Table 3 lists various components and measurements that were handled on a non-regular basis during the study in order to get a more comprehensive assessment of the exposure atmospheres. Procedural details are given in the following discussion of the results of the measurements.

Analytical Results of the Study

Automatically Monitored Components

The mean concentration values for the major components during the 5+-year period of the study are listed in Table 4. A comparison of the data with the nominal concentration values listed for the start of the study shows a satisfactory achievement of the intended levels. A running account of the chamber atmosphere monitoring is shown in Figure 4 of Chapter 1. It is evident that the concentrations were maintained reasonably constant throughout the study.

Specific Hydrocarbons

The gas chromatographic system to monitor specific organic components in the exposure atmospheres was put into service 9 months after the start of the study and carried on to the end. The system was used to measure various C_2 to C_5 hydrocarbons in 6 to 8 daily samples collected from exhaust atmospheres after chamber concentrations reached equilibrium. Table 5 summarizes typical concentrations. Normal variations for these components are indicated in parentheses.

Chamber samples were also analyzed for aromatic hydrocarbons. The two compounds determined in this phase, and their typical concentrations in exhaust chambers, were benzene

Table 3. Pollutants Measured Periodically

Component	Chamber type(s)	Frequency	Method of analysis
Hydrocarbons	I, R, I + SO_x, R + SO_x	Daily, as of May, 1966	Gas chromatography
Lead (Pb)	I, R, I + SO_x, R + SO_x	Periodic (weeks at a time)	A.A. spectro-photometry
Particulate mass	all	Periodic (weeks)	Phototape sampler
Particulate size	all	Periodic (weeks)	Optical, light scattering
Particulate anions	I, R, I + SO_x, R + SO_x	One period of several weeks	
Sulfate			Wagman, 1967 (2)
Nitrate			Saltzman, 1954 (1)
Halide			Bergmann, 1957 (3)
Acrolein	I, I + SO_x	Periodic (weeks)	Cohen, 1961 (4)
Formaldehyde	I, I + SO_x	6-mo. period in 1966	Sawicki, 1961 (5)

Table 4. Pollutant Concentrations in the Exposure Chamber Atmospheres During the Chronic Exposure Study (ppm [mg/m^3], mean ± s.d.)

Group	Atmosphere	Carbon monoxide CO	Hydrocarbons (as CH_4)	Nitrogen dioxide NO_2	Nitric oxide NO	Oxidants as O_3	Sulfur dioxide SO_2	Sulfuric acid H_2SO_4
1	Control air (CA)	4.9	2.7	0.04	0.04	0.02	0.03	....
2	Non-irradiated auto exhaust (R)	97.5 ± 10.0 (112.1 ± 11.5)	27.5 ± 4.4 (18.0 ± 2.9)	0.05 ± 0.02 (0.09 ± 0.04)	1.45 ± 0.42 (1.78 ± 0.52)	....	....	....
3	Irradiated auto exhaust (I)	94.5 ± 19.6 (108.6 ± 22.5)	23.9 ± 6.1 (15.6 ± 4.0)	0.94 ± 0.36 (1.77 ± 0.68)	0.19 ± 0.29 (0.23 ± 0.36)	0.20 ± 0.09 (0.39 ± 0.18)	....	....
4	$SO_2 + H_2SO_4(SO_x)$	....	....	....	....	....	0.42 ± 0.22 (1.10 ± 0.57)	0.02 ± 0.01 (0.09 ± 0.04)
5	Non-irradiated A.E. with $SO_2 + H_2SO_4(R + SO_x)$	98.4 ± 13.8 (113.1 ± 15.9)	27.4 ± 4.3 (17.9 ± 2.8)	0.05 ± 0.03 (0.09 ± 0.06)	1.51 ± 0.44 (1.86 ± 0.54)	....	0.48 ± 0.23 (1.27 ± 0.61)	0.02 ± 0.01 (0.09 ± 0.04)
6	Irradiated A.E. with $SO_2 \pm H_2SO_4(I + SO_x)$	94.8 ± 19.8 (109.0 ± 22.8)	23.9 ± 6.0 (15.6 ± 3.9)	0.89 ± 0.36 (1.68 ± 0.68)	0.19 ± 0.29 (0.23 ± 0.36)	0.20 ± 0.08 (0.39 ± 0.16)	0.42 ± 0.21 (1.10 ± 0.56)	0.03 ± 0.01 (0.11 ± 0.04)
7	Nitrogen oxides (high NO_2), ($NO_L + NO_{2H}$)	....	....	0.64 ± 0.12 (1.21 ± 0.22)	0.25 ± 0.06 (0.31 ± 0.08)	....	....	....
8	Nitrogen oxides (high NO), ($NO_H + NO_{2L}$)	....	....	0.15 ± 0.33 (0.27 ± 0.62)	1.67 ± 0.21 (2.05 ± 0.26)	....	....	....
	Nominal	100	24-30	0.5-1.0	1.5-2.0	0.2-0.4	0.50	(0.100)

Table 5. Hydrocarbon Concentrations in the Exposure Atmospheres at Equilibrium (ppm)

Components	Treatment			
	R	I	R + SO$_x$	I + SO$_x$
C_2H_4-C_2H_6	1.04 (0.25)	0.86 (0.19)	1.00 (0.25)	0.86 (0.18)
C_2H_2	0.36 (0.10)	0.34 (0.07)	0.37 (0.09)	0.35 (0.08)
C_3H_8	0.02 (0.02)	0.02 (0.02)	0.02 (0.02)	0.02 (0.02)
C_3H_6	0.29 (0.09)	0.15 (0.06)	0.26 (0.10)	0.17 (0.08)
i-C_4H_{10}	0.05 (0.03)	0.04 (0.02)	0.05 (0.02)	0.05 (0.02)
n-C_4H_{10}	0.21 (0.05)	0.20 (0.05)	0.20 (0.08)	0.20 (0.08)
Butene-1	0.02 (0.03)	trace (0.01)	0.03 (0.03)	0.01 (0.01)
Isobutylene	0.02 (0.02)	trace (0.01)	0.02 (0.02)	trace (0.01)
Butadiene	0.02 (0.04)	trace (0.01)	0.04 (0.04)	trace (0.01)
i-C_5H_{12}	0.27 (0.07)	0.24 (0.10)	0.26 (0.10)	0.25 (0.11)
n-C_5H_{12}	0.15 (0.05)	0.14 (0.06)	0.14 (0.06)	0.15 (0.05)

(0.1 ppm) and toluene (0.2 ppm). Several other peaks were observed in the chromatogram, but generally at concentrations too low to be adequately quantified.

At various times analyses were made for other aliphatic hydrocarbons. Concentrations of hexane, cyclohexane, heptane and octane were estimated at about 0.1, 0.1, 0.05, and 0.1 ppm, respectively. Separations were poor among this group and the results should be considered semi-quantitative only.

Atmospheric Particulate Concentration and Size

Relative values of airborne particulate concentrations and of particle size were obtained to present a comparison among the different exposure atmosphere types. A phototape sampler was used for indicating the mass concentration. It measured and recorded the light transmission through an area of filter paper (tape) on which a thin layer of particulate had been collected. The decrease in transmission over the collection period represented the equivalent of a concentration of airborne particulate. The phototape sampler was not calibrated to indicate an absolute mass concentration but was used for a limited period for comparisons of particulate loading among the chambers. The transmission values reported in Table 6 show the final percent transmission after sampling 15 cubic feet over a 30-minute period onto a 14-mm-diameter spot, with the initial transmission adjusted to 100%. The data show that the transmission of the I-chamber samples was the lowest (i.e., indicating the highest concentration of particulates), followed in order by R, SO$_x$, NO$_x$ and clean air.

An optical particle sizer using the light scattering principle indicated that more than 90% of the particles of any atmosphere type were smaller than 0.5 μm in diameter. The I atmospheres bore 10 to 15 times as many particles as the R atmospheres. The CA chambers contained only about one percent the number of particles found in the I-chamber atmospheres.

Table 6. Phototape Sampler Transmission Values

Atmosphere type	Transmission (%)
Irradiated auto exhaust (I)	85.5
Irradiated A.E. + SO_2 + H_2SO_4 (I + SO_x)	88.7
Non-irradiated A.E. (R)	93.0
Non-irradiated A.E. + SO_2 + H_2SO_4 (R + SO_x)	95.1
SO_2 + H_2SO_4 (SO_x)	97.8
NO + NO_2 (NO_x)	99.4
Clean air (CA)	100.0

Analysis of the particulates collected by filters (6) gave the concentrations of anions shown in Table 7.

Table 7. Anions Associated with Airborne Particulate (mg/m^3)

	R	I	R + SO_x	I + SO_x
Sulfate	0.37	5.9	21.0	99.2
Nitrate	3.3	581.	11.3	223.
Halide	20.2	28.7	17.6	32.7

Acrolein

Acrolein was determined by the method of Cohen and Altshuller (4), which utilizes 4-hexylresorcinol as the color-producing agent. The method measures concentrations as low as 0.001 ppm. A total of 150 samples from I chambers 14, 16 and 22, and I + SO_x chambers 7, 15 and 17 were analyzed in 1966 and 1967. The data are summarized in Table 8.

Table 8. Acrolein (ppm)

Treatment	Min.	Max.	Mean
I	0.0090	0.0288	0.0198
I + SO_x	0.0072	0.0306	0.0192
All samples	0.0072	0.0306	0.0195

Formaldehyde

Formaldehyde analyses were performed on 357 total samples from I chambers 14, 16 and 22 and I + SO_x chambers 7, 15 and 17 in 1966, using the 2-hydrazinobenzothiazole method of Sawicki *et al.* (5). Table 9 summarizes these data.

Table 9. Formaldehyde Concentrations (ppm)

Treatment	Range		
	Min.	Max.	Mean
I	0.28	1.00	0.57
I + SO_x	0.26	0.88	0.49
All samples	0.26	1.00	0.53

Atmospheric Lead Determinations

Atmospheres containing raw and/or irradiated auto exhaust mixed with SO_x were analyzed to determine chamber atmospheric lead concentrations during 1967, 1969 and 1970 (7). The sampling plans for the three determinations were somewhat different from each other. In 1967 only total lead in four treatment atmospheres was measured by a direct method. In 1969 assays for both soluble and insoluble lead were performed and total lead concentrations were calculated. In 1970, both calculated and direct assays were compared by means of paired filters, but only two treatments were sampled.

Filter holders connected with calibrated critical pressure orifices were used to collect 16-hour suspended-particulate samples from the automobile exhaust chambers on two-inch GA-4 grid Millipore filters having a pore size of 0.08 μ. Glass probes were introduced to the chambers to sample at a point directly above the animals.

Both water and acid-lead extracts were analyzed by atomic absorption spectrophotometry. Lead absorption was measured at a wave length of 283 μ.

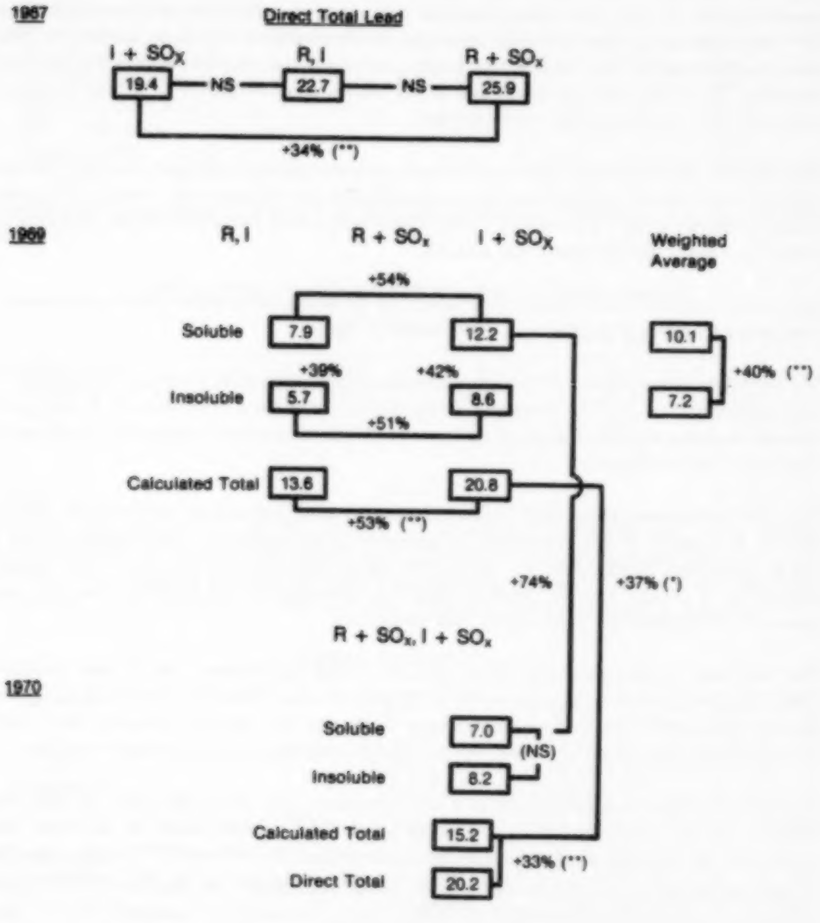
The 1967 data showed about 30% higher average calculated total lead concentrations in R + SO_x, compared to I + SO_x. A corresponding difference did not exist between R and I. All four atmospheres exhibited large, positively correlated, concomitant day-to-day variations in total lead concentrations.

The 1969 data showed about 50% higher concentrations of both soluble and insoluble lead in the I + SO_x and R + SO_x treatments as compared to the respective concentrations in the I and R treatments. Soluble lead was about 40% higher than insoluble lead in all four exhaust treatments. A similar soluble:insoluble ratio was observed for CA and SO_x but was not measured as precisely as for the other four treatments.

The 1970 data showed an almost equal division of total lead between soluble and insoluble forms. No treatment differences existed for either of the lead forms, for their total, or for the directly measured total lead concentrations. However, the directly measured total lead concentrations were about one-third higher than the calculated total lead concentrations.

Averages of directly measured total lead concentrations were about the same in 1967 and 1970 for the I + SO_x and R + SO_x treatments. On the other hand, for the same two treatments, the average calculated total lead concentration for 1969 was 37% higher than the average calculated total lead for 1970. The difference appeared to be due entirely to the soluble lead, which was 74% higher in 1969.

Figure 1 summarizes these conclusions. Averages are shown wherever components of the average were statistically homogeneous.


1967
Direct Total Lead
I + SO$_x$
R, I
R + SO$_x$
19.4
NS
22.7
NS
25.9
+34% (**)
1969
R, I
R + SO$_x$
I + SO$_x$
Weighted Average
+54%
Soluble
7.9
12.2
10.1
+39%
+42%
+40% (**)
Insoluble
5.7
8.6
7.2
+51%
Calculated Total
13.6
20.8
+53% (**)
+74%
+37% (*)
R + SO$_x$, I + SO$_x$
1970
Soluble
7.0
(NS)
Insoluble
8.2
Calculated Total
15.2
+33% (**)
Direct Total
20.2

(NS) = not statistically significant at P = 0.05

(*) = statistically significant at P = 0.05

(**) = statistically significant at P = 0.01

Figure 1
Pattern of differences in mean CAE study lead concentrations (µg/m^3)

References

1. Saltzman, B.E. 1954. Colorimetric microdetermination of nitrogen dioxide in the atmosphere. Anal. Chem. 26: 1949-1955.
2. Wagman, J., R.E. Lee, and C.J. Axt. 1967. Influence of some atmospheric variables on the concentration and particle size distribution of sulfate in urban air. Atmos. Environ. 1: 479-489.
3. Bergmann, J.G., and J. Sanik. 1957. Determination of trace amounts of chlorine in naphtha. Anal. Chem. 29: 241-245.
4. Cohen, I.R., and A.P. Altshuller. 1961. A new spectrophotometric method for the determination of acrolein in combustion gases and the atmosphere. Anal. Chem. 33: 726.
5. Sawicki, E. 1961. The 3-methyl-2-benzothiazolone hydrazone test. Anal. Chem. 33: 93.
6. Lee, R.E. 1971. Concentration and particle size distribution of particulate emissions in automobile exhaust. Atmos. Environ. 5: 225-237.
7. Barkley, N.P., 1972. The concentration of lead in automobile exhaust exposure chambers. AIHA Journal 33: 678-683.

Asilomar Conference Discussion

Malanchuk: If you will refer to Table 6, the phototape sampler transmission values show a progressive increase in the transmission value, which means that at the top of the list with the lower transmission, that actually means a higher relative concentration of particulate mass. You can see it stepwise there from 85.5 for the irradiated auto exhaust to 100.0 for clean air.

Tyler: The most particles are in the irradiated, and they literally grew there as you irradiated it, pretty significantly over the nonirradiated.

Malanchuk: Right.

Question: Is there a difference in the aging time in the different atmospheres? In other words, is there more time for particulate growth in the irradiated?

Malanchuk: Yes. There was very little time for the raw exhaust particulate due to the direct dilutant effect versus the irradiated particulate in the irradiation chambers.

Stara: We have realized this problem in our more recent studies, and we now have a mixing chamber for the raw exhaust also, which gives us the same residence time for the various chemical reactions in both raw and irradiated exhausts.

Lewis: Mr. Malanchuk, I have heard criticisms of the study, many of which were invalid and some of which I consider valid. Can you make any kind of quantitative estimate as to how much of the particulate was lost in our simulated system versus what one might encounter in the atmosphere? Was it 10%, 50%, 80%, or what? Dr. Stara is nodding his head yes; maybe he can answer that.

Stara: I don't know if Mr. Malanchuk momentarily recalls, but we have done a study of this problem. The losses of particulate from the mixing chamber or the irradiation chamber to the animal chamber are between 10 and 20%.

Malanchuk: Right.

Lewis: What you are trying to simulate is the urban environment where you have dilution as it comes out of the tailpipe. Well, the criticism that I heard was that we didn't have enough particulate, and intuitively I would think that we had more than would be found in the urban environment.

Question: How many changes per hour did you have in the chamber?

Malanchuk: Fifteen.

Comment: There's one point; maybe I missed it in your discussion of the exposure system, Dr. Stara. It is my understanding that on an engine that is pulling weight, the exhaust would be different than on an engine that is free-wheeling. I heard you mention something about the simulated weight or wear. But was it actually pulling any weight?

Stara: Yes. The stationary dynamometer system that we employed attempts to simulate the road conditions.

Hueter: Engine output, and that's why it was cycling, because you don't get much NO_x in certain modes of operation without cycling the engine.

Stara: For example, during steady speed this may be so. We had to use an approved engine cycle which decelerates and accelerates to produce auto exhaust which has the required pollutant combination.

Hueter: Relative to lead content, I wondered, the concentrations were similar, but were the particle sizes similar, and do you have some data on the ratio of organic/inorganic, which were different?

Malanchuk: I don't know whether I have all the raw data here to readily answer your questions. I think the report that we had written by Barkley *et al.* (7) shows a fairly detailed discussion of this problem. I would refer you to that, to answer your question.

Lewis: I recall from the biological data that there was a difference in bone meal which we attributed to particulate size, chemical composition, and solubility.

Malanchuk: As I remember it, there seemed to be a lower lead concentration in the SO_x chambers, wasn't there? Can you remember whether you saw that in the Barkley report? It's as if the SO_x had a chance to do a scavenging act, apparently, on the lead and bring it down.

Hueter: Although the animals in raw and irradiated exhaust received the same essential lead exposure, measured as total lead, the animals receiving the lead in raw exhaust had higher blood levels than those receiving irradiated exhaust. We didn't know if that was due to particle size or due to chemical form.

Stara: There are two or three future studies that have not been completed, even though for all practical purposes I feel that the major study is completed. One of them is the bone lead values at sacrifice. We still have the bone samples. As a matter of fact, Webster Jee from Salt Lake City suggested a small amount of lead mapping in the bone to see the lead distribution in the hydroxyapatite.

Albert: Could you explain what Figure 1 is about.

Malanchuk: These values and percentages are to show the difference between conditions that were similar, and that the values are apparently significantly different, such as the 37%(*). For instance, if you look at the 40%(**) on the extreme right-hand side, it says "statistically significant at $P = 0.01$," meaning that between the soluble and insoluble forms of lead that were analyzed in 1969, the 10.1 and 7.2 show that much difference, to that extent.

Hueter: Right to the left of that you've got "R, I" and "$R+SO_x$, $I+SO_x$." So you say that "R, I" means soluble/insoluble with 7.9/5.7. There wasn't any difference between R and I.

Malanchuk: The weighted average for the solubles (R and I plus the $R+SO_x$ and $I+SO_x$) is shown statistically to be significant at $P = 0.1$ (**) when compared to the composite value for the insolubles, however. The R did not vary significantly from the I values in these particular groupings of data, it would seem.

Hueter: We didn't have time to find out why. If lead comes out of the tailpipe, it's usually in a form of chlorobromo-lead, either di-chloro, di-bromo, or chloro-bromo, something like that. I don't know if the irradiation of that has an effect on changing the form before it gets to the animal.

Bhatnagar: But you probably have more sulfate in the irradiated exhaust.

Albert: But was there more lead in the nonirradiated than in the irradiated atmospheres?

Hueter: Not significantly, according to this table here.

Albert: You can make that out?

Hueter: Well, it says R, I, 22.7, so apparently they were so close that they weren't significant.

Albert: What is the 22.7?

Hueter: $\mu g/m^3$, I presume.

Malanchuk: Right.

Albert: Nonirradiated and irradiated. Is there a difference?

Malanchuk: No.

Hueter: Not in total lead.

Albert: How can you tell? What about the holdup in the radiation chamber where you could lose particles?

Hueter: The particle size at that point where it comes out of the tailpipe is pretty small.

Lewis: And it's diluted equivalent to the dilution to 100 ppm of CO.

Question: In the case of sulfate in the irradiated versus the nonirradiated atmospheres there is a difference of 6 μg.

Lewis: That's what Mr. Malanchuk said. The sulfates seem to be scavenging lead.

Hueter: But yet if that's true, then more of it should be insoluble than soluble, and it's not.

Lewis: But we don't know what kinds of subsequent chemical reactions occurred within the irradiation chamber.

Busch: It was significant that the value for R and I had enough measurement error to be potentially equal to either the 19.4 or 25.9. There wasn't a statistical difference in either of them. But the two extremes, the $I + SO_x$ and the $R + SO_x$, were different.

Malanchuk: These samples were done in three different years — 1967, 1969, and 1970 — and we may have had differences in engine operations and fuel supply that may have affected the lead and particulate analytical scheme. So that, looking at the overall picture, I come up with a couple of bare conclusions. One is that over that period of 1967-1970, and I am repeating myself, the lead concentration apparently had no radical change in the gasoline supply during that period of time. Take it for what it's worth. Apparently the SO_x had a kind of scavenging effect on bringing down the lead since there seemed to be a fairly good difference in the SO_x chambers as compared to those that did not have SO_x supplement.

Lewis: Correct me if I'm wrong, but I think it's important that when you work for the government, GSA wants you to buy on low bid, so we didn't always have the same supplier. Isn't that right, Dr. Hueter?

Hueter: That's true. We didn't have storage facilities to store enough gasoline for 5 years.

Lewis: The blend changed through those years, as did other factors which affected the composition of the gasoline used to generate the auto exhaust atmospheres.

Busch: The engine was overhauled and tuned up, rings and valves were replaced, the head was taken off periodically and the valves ground — wasn't there a complete new engine installed during the course of the study at one time? I can't recall.

Hueter: There were two engines. They served as alternates to one another. When one was being overhauled, the other one was being run.

Busch: But we never purchased new engines, did we?

Malanchuk: No. Not for this study as far as I know.

Lewis: Engine changes only occurred in the old initial study when things were changed around for the new system, i.e., 1972.

Busch: What bothered me was that the NO seemed to be so stable around the target level during the major part of the study and then dropped off some tenfold in the last couple of years. I wondered if we could pinpoint why that happened. The NO_2 remained the same, but the NO is lower. Could it have been an analytical error?

Malanchuk: Let me appeal to Dr. Stephens here, as an expert. Aren't we put out yet to explain the complex interactions among the NO_2, NO, oxidants and so forth in the atmosphere? There is a very touchy condition where you have this equilibrium, NO_2, NO, and ozone, and hydrocarbons involved. I think with a slight decrease in hydrocarbons or what have you, you can have a pretty harsh change in the other components.

Busch: Are the 6-month averages plotted in the diagram (Figure 4 of Chapter 1)?

Malanchuk: I think they're more like about 4-1/2 or 5 months.

Busch: In the computerized data analysis, we were assessing the values each 6 months.

Malanchuk: This was the data that was handled especially by the group summarizing the information for the final report.

Comment: Well, to establish that curve, I think that special statistical or data accumulation studies were conducted.

Stephens: Well, I couldn't claim to be an expert as far as the dynamics between the gases are concerned. We've had a great deal of experience with NO_2 compound exposures as well as ozone. I do not claim any expertise in the dynamics between the two other atmospheres.

Lewis: I don't know what to attribute it to, but it's only in one of the eight treatments where it's outstanding. That's the $I + SO_x$. In the other six treatments, it's not apparent. If you look at Figure 4 of Chapter 1, which is the auto exhaust and auto exhaust + SO_x, it's essentially linear. If you look at the I, it's not nearly as abnormal, although the concentrations of oxides of nitrogen are rising in the last phase of 1971. So whether it's an analytical mistake or whether it's an engine or what, it's hard to say. But it would appear to be related to an irradiation interaction phenomena.

Hueter: It couldn't be an engine because the other chambers also got the same exhaust from the same engine. So it would have to be an analytical error or some other reaction occurring in the system.

Lewis: But I think it's more of an artifact than a reality.

Hueter: You'll never explain it.

Bhatnagar: It's not an artifact if you start looking for all the chemical entities and free radical species in the irradiated exhaust.

Lewis: Yes, but to tie them into the NO levels is very difficult when you look at all other things in common, the CO, the NO_2, etc.

Bhatnagar: Dr. Stara, I have a question for you. You said that in your later experiments, the raw exhaust also passed through a chamber giving it the same time as irradiated exhaust. Is there a difference in particulate concentration?

Stara: First of all, this phenomenon was not observed during this study. It was noted during the studies of catalytic converter emissions. And in the latter instance, the particulate matter analysis is completely different. The particles are inorganic sulfuric acid and sulfates rather than the inorganic/organic particulate mix you find without catalytic converters. So I don't think that the question is relevant to this study.

Hueter: Also, the irradiation there creates particulate matter. You always have more particles in irradiated than in raw.

Stara: Yes.

Busch: If I could make one observation about the quality control on the atmospheric measurements in the chambers; with respect to the CO concentrations, which I understand were nonreactive, we used data during an 8-hour day, monitoring data. We had 5-minute or 10-minute readings, as I recall, automatically monitored, and they were plotted. A calculation was made completely independent of the data. An independent mathematical model was developed based on a differential equation. The differential equation showed that the rate of increase in the CO was proportional to the rate of introduction of CO into the chamber. The input concentration (it was monitored going into the radiation chamber), the flow rate, and the volume of the chamber were known (it was 864 cubic feet, as I recall). Based on the differential equation we obtained a mathematical model for the exponential increase in CO concentration in the irradiation chamber. Assuming that there was instantaneous mixing and that as the material enters it immediately mixes and is diluted with what was already in the chamber, we could draw the mathematical curve which followed almost exactly through the data. It was remarkable. It indicated to us that the engineering of the radiation chamber, including the fans to mix the gases as they were introduced, was adequately done.

3. CLINICAL SUMMARY OF BEAGLE STUDY. PHYSICAL EXAMINATIONS, OCULAR EXAMINATIONS, HEMATOLOGY EXAMINATION (POST-EXPOSURE) AND BLOOD CHEMISTRIES

J.G. Orthoefer, J.F. Stara, Y.Y. Yang and K.I. Campbell

Clinical examinations were periodically conducted by a veterinarian to assess the health status of the animals. The results of these examinations were used to form a basis for therapy and/or for inclusion or exclusion of functional data to be analyzed.

During the exposure period, the use of drugs was minimized, particularly drugs which might change the animals' response to a pollutant, such as corticosteroids. Health disorders were cared for immediately. The primary purpose of any treatment was to prevent clinical disorders from compromising or complicating the toxicologic interpretations.

Body weights were taken on a monthly basis throughout the study. The body weights were corrected on a body volume basis using girth size at the shoulders to determine an ideal weight. No group differences in body weight were attributed to the treatment regimen. However, for intervals of time throughout the study period certain animals had to be specially fed in order to adjust the body weight to a more ideal weight. Cases of obesity and cachexia were corrected primarily by these husbandry practices. This was accomplished by removing the animals at night during the normal feeding time and allowing them to eat prescribed amounts of food.

Dermatitis cases were seen throughout the exposure and post-exposure period. The cases ranged from mild to severe (Figure 1). There were specific exposure trends in dermatitis with a prevalence in the NO_L + NO_{2H} group in 1969 and 1971. However, a relatively high prevalence occurred in the control group. Therefore, it was difficult to attribute the dermatitis to anything other than some unknown factor. No specific etiology was ever determined. The dictates of the study demanded that four beagle dogs live in an area of 9 square feet. This may have been more detrimental psychologically to the animals than exposure to pollutants. By December of 1973, as shown in Figure 1, the dermatitis was of relatively minor significance, indicating that removal from the chambers and placement in a less restricted environment during non-exposure periods was beneficial. Since the animals were removed to a different environment entirely (food, caging, disinfectants, etc.), the change in skin condition cannot be attributed solely to a lack of pollutant exposure. The cases of dermatitis were vigorously treated after the cessation of the exposure, when time was available for therapeutic baths, etc. Although higher incidences of dermatitis occurred in certain groups during the exposure period, and these effects persisted in some groups to a minor degree even during the recovery period (Groups 6 and 7, Figure 1), no attempt was made to attribute the dermatologic differences directly to the pollutants.

Ocular examinations were performed during the routine physicals. Observations were made of the external and internal structures of the eye in search of gross disorders. Two more detailed examinations were conducted, one by Dr. Milton Wyman (Ohio State University) near the termination of the exposure, and one by the University of California during the post-exposure period just prior to euthanasia.

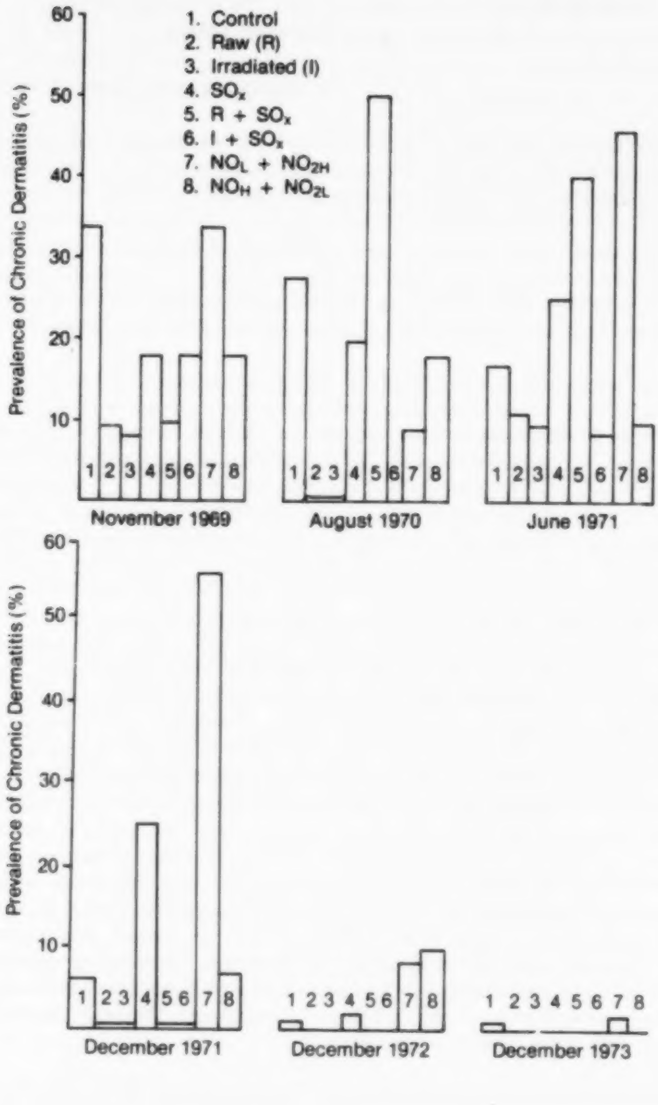


Figure 1
Prevalence of chronic dermatitis in experimental animals.

Epiphora (constant washing of the eye by tears) may have been related to the exposure atmospheres. As shown in Figure 2, the dogs exposed to a few atmospheres (2, 3, 6) show a prevalence of epiphora above that of the control group of dogs. This relationship did not persist after the cessation of exposure.

The tabulated results of the detailed ocular examination prior to cessation of exposure are shown in Table 1. Although some superficial effects due to close contact of the pollutants with the eye were expected, no such effects were discerned. Permanent eye damage may have been prevented by the constant washing of the eye associated with epiphora.

No association between exposure and oral disorders or otitis was observed. Dental calculi and periodontal disorders were largely prevented by routine cleaning of the teeth by the caretaking staff. Otitis externa was treated by the use of antibiotic ointments when necessary.

From the initiation of the experiment, hematological studies were conducted at approximately 6-month intervals. Measurements included red (RBC) and white (WBC) blood cell

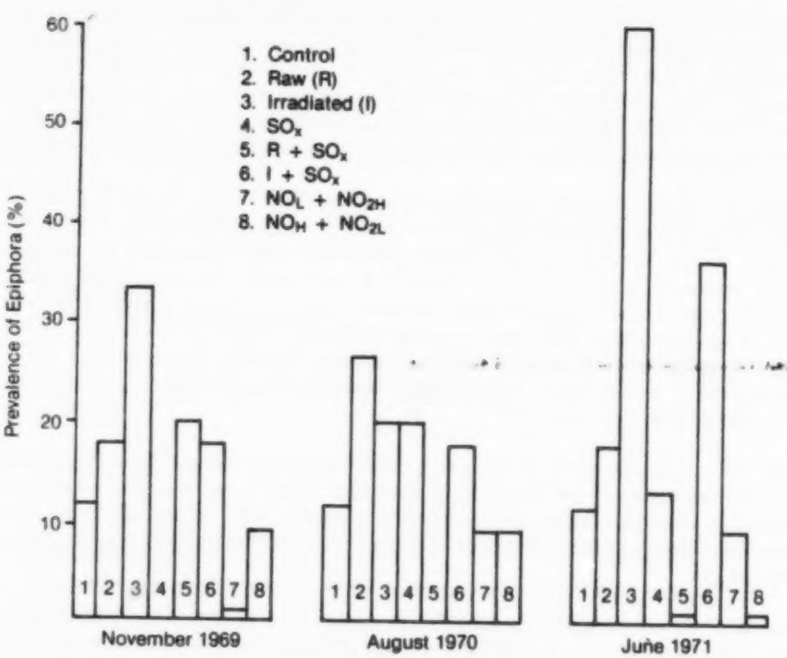


Figure 2
Prevalence of epiphora in experimental animals.

Table 1. Tabulation of Ocular Lesions, as Reported by Dr. Wyman

N[a]	Exposure	Lids	Con-junct.	Cornea	Iritis	Lens	Retina	Other
18	CA	3	3	3		3		Papill 1
11	R	2	2	2	1	2	1	Papill 1
10	I	1				2		Phthisis bulb
8	SO_x	2	1			2	1	
10	$R + SO_x$	4		2		4		
11	$I + SO_x$	4		3		6	1	
11	$NO_L + NO_{2H}$	2	1	2		4		
10	$NO_H + NO_{2L}$	2		1		1		Papill 1
89	**Total**							

Plus: a. Dental abscess-related inflammation — one dog ($R + SO_x$)

b. Post-distemper-related retinal atrophy — one dog ($NO_H + NO_{2L}$)

[a] *Two dogs (one in SO_x and one in $R + SO_x$) were not accounted for in the report as having been examined. They are assumed to have been in the "normal aging changes" category.*

counts, hematocrit (Hct), hemoglobin (Hg), and the derived indices of mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH). The exposure hematology did not include differential cell counts. Separate univariate analyses of variance of hematologic data were performed for each measurement period. Whenever significant treatment effects were detected, twelve defined contrasts among the treatments were tested for nullity to reveal a possible cause of the effects.

During the exposure, groups exposed to auto exhaust (R, $R + SO_x$, I, and $I + SO_x$) had higher mean Hct and Hg values than those exposed to no exhaust (CA, SO_x, $NO_L + NO_{2H}$, $NO_H + NO_{2L}$). The RBC of auto exhaust groups was higher throughout the study, even though statistical significance was not obtained at every period. The comparison of Hct, Hg and RBC under exhaust and no-exhaust conditions is shown in Figures 3 and 4. There was no effect of auto exhaust on WBC, and no consistent effect on MCHC, MCV or MCH. The I and $I + SO_x$ groups showed reduced RBC and Hg after exposure was terminated. No post-exposure effect on any of the hematological parameters was detected in any of the other treatment groups.

Table 2 presents a summation of the post-exposure hematology results. This work was done in Dr. Jain's laboratory at the University of California, Davis. A more detailed post-exposure hematology statistical work-up is presented in Appendix I of this chapter. The post-exposure hematology included the differential cell count and differed in this respect from the exposure hematology.

A set of 19 clinical chemistry parameters was measured on blood samples taken four times during the study. These analyses were performed by Automated Analytical Laboratories, Inc. (AAL) in Ventura, California. Samples were obtained in special tubes or bottles and shipped air mail to AAL three times from Cincinnati, Ohio and once just prior to euthanasia from Davis, California. Although there were some deviant individual values, these did not reflect consistent or statistically significant group trends, and were not regarded as toxicologically significant. The group mean values for each of these tests are appended to this report (see Appendix II).

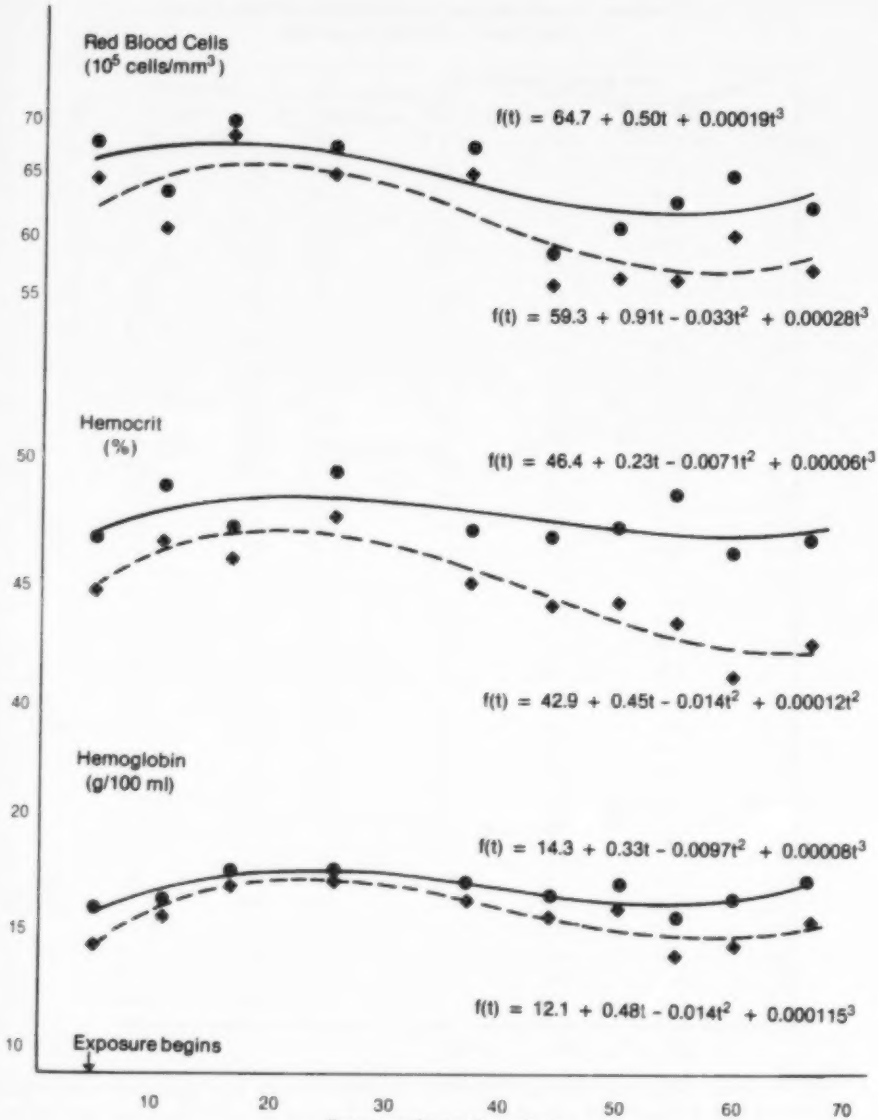


Figure 3
The effect of exposure to auto exhaust on blood indices in the beagle dogs
pre-exposure and exposure. (Solid curves represent CO atmospheres, dashed are non-CO.)
◆ = Non-exhaust
● = Exhaust

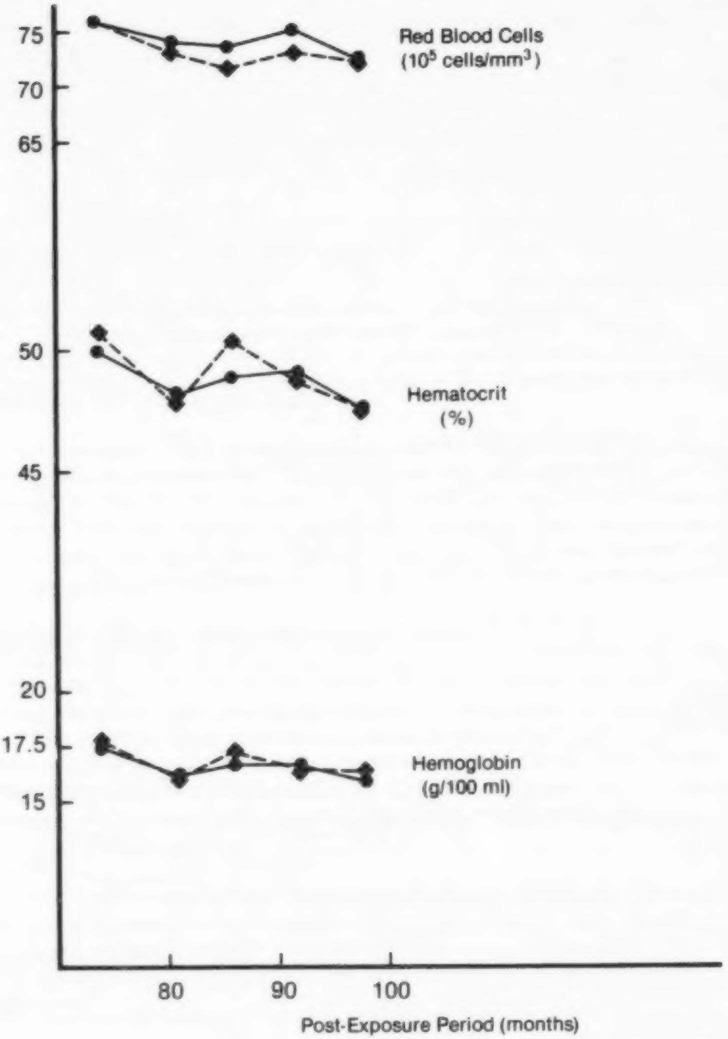


Figure 4
The effect of exposure to auto exhaust on blood indices in the beagle dogs post-exposure. (Exposure ended at 68 months.)
◆ = Non-exhaust
● = Exhaust

Table 2. Certain Hematological Abnormalities in Beagles in Relation to Different Treatments

Period	Treatment and No. of dogs		Percentage of dogs with:				
			Nucleated RBC	Plasma proteins >6 g%	Plasma proteins <6 g%	Left shift 300 Bands	Basophils <0.5%
Nov 1971	CA	(18)	38.9	5.5	0.0	33.3	66.6
	I	(10)	40.0	0.0	0.0	50.0	50.0
	I + SO_x	(11)	18.2	27.3	9.1	45.5	45.5
	R	(11)	45.5	27.3	0.0	36.4	54.5
	R + SO_x	(10)	60.0	10.0	0.0	30.0	70.0
	NO_x	(20)	75.0	5.0	0.0	15.0	60.0
	SO_x	(8)	25.0	12.5	0.0	25.0	25.0
June 1972	CA	(17)	17.6	0.0	5.9	17.6	55.0
	I	(10)	33.3	0.0	10.0	22.2	44.0
	I + SO_x	(11)	27.3	0.0	9.1	27.3	45.5
	R	(11)	27.3	0.0	0.0	36.4	72.7
	R + SO_x	(10)	10.3	0.0	0.0	30.0	90.0
	NO_x	(20)	35.0	0.0	0.0	40.0	65.0
	SO_x	(8)	0.0	0.0	0.0	25.0	50.0
Nov 1972	CA	(17)	23.5	0.0	0.0	5.9	76.4
	I	(10)	30.0	0.0	0.0	0.0	80.0
	I + SO_x	(11)	27.3	0.0	0.0	18.2	45.5
	R	(10)	30.0	10.0	0.0	0.0	60.0
	R + SO_x	(10)	30.0	0.0	0.0	30.0	90.0
	NO_x	(20)	35.0	0.0	0.0	30.0	60.0
	SO_x	(8)	0.0	0.0	0.0	25.0	75.0
May 1973	CA	(17)	35.3	0.0	0.0	23.5	76.5
	I	(10)	20.0	0.0	10.0	10.0	60.0
	I + SO_x	(11)	18.2	0.0	18.2	45.5	63.6
	R	(10)	30.0	0.0	0.0	20.0	70.0
	R + SO_x	(10)	20.0	0.0	0.0	30.0	80.0
	NO_x	(20)	25.0	0.0	0.0	25.0	65.0
	SO_x	(8)	0.0	0.0	0.0	12.5	87.5
Nov 1973	CA	(17)	23.5	5.9	0.0	11.8	67.7
	I	(10)	40.0	0.0	0.0	0.0	50.0
	I + SO_x	(11)	18.2	9.1	0.0	0.0	36.4
	R	(10)	20.0	20.0	0.0	0.0	50.0
	R + SO_x	(10)	20.0	0.0	0.0	0.0	50.0
	NO_x	(20)	20.0	5.0	5.0	5.0	50.0
	SO_x	(8)	25.0	0.0	0.0	0.0	37.5

Anaesthetic accidents and trauma resulting from fighting were the major causes of pre-euthanasia deaths during the study. Tissue obtained from the dead animals was generally inadequately preserved, thus preventing meaningful morphological examination. However, in most cases the cause of death could be verified. Table 3 shows the number and causes of pre-euthanasia deaths.

Table 3. Date and Cause of Pre-Euthanasia Deaths in the Chronic Auto Exhaust Study

Treatment	Month/year of death	Cause of death[a]
Control	6/67	A_1
	2/70	T
	12/71	A_1
Raw exhaust (R)	5/68	T
	6/72	X
Irradiated exhaust (I)	12/69	T
	12/69	T
SO_x	12/68	A_1
	4/70	T
	8/70	A_1
	4/71	A_2
R + SO_x	10/68	A_1
	5/66	X
	2/74	X
I + SO_x	10/68	A_1
$NO_L + NO_{2H}$	12/69	T
	11/71	T
$NO_H + NO_{2L}$	3/67	A_1
	2/71	A_1

[a] *A_1 = Anesthesia related to obtaining physiological data*
A_2 = Anesthesia related to surgical procedure for medical treatment
T = Trauma usually resulting from fights
X = Other

Appendix I

Means by Treatment (Nov. 1971)

	CA	R	I	SO_x	$R + SO_x$	$I + SO_x$	$NO_L +$ NO_{2H}	$NO_H +$ NO_{2L}
TP	7.206	7.409	7.340	7.225	7.450	7.453	7.100	7.080
RBC	7.719	7.570	7.700	7.805	7.641	7.412	7.324	7.595
HG	17.835	17.209	17.690	18.513	17.880	17.100	17.270	17.640
PCV	52.382	50.455	50.700	52.250	51.300	49.182	49.900	51.000
MCV	67.007	66.623	66.229	67.122	67.137	66.515	68.203	67.225
MCHC	34.280	34.065	34.895	35.424	34.879	34.548	34.557	34.581
WBC	11594	11754	10350	10075	12850	10772	12170	10940
BAND	833	553	291	230	933	267	312	172
NEUT	6595	6994	6051	5685	6644	6794	7111	5705
LYM	2743	2673	2504	2755	2710	2379	2933	2943
MONO	977	935	812	938	992	1029	1033	808
EOS	361	326	256	365	384	266	672	387

Key:
TP = Total protein (g/100 cc)
RBC = Red blood cell count (x 10^6)
HG = Hemoglobin (g/100 cc)
PCV = Packed cell volume (%)
MCV = Mean corpuscular volume
MCHC = Mean corpuscular hemoglobin content
WBC = White blood cell count
BAND = Immature polymorphonuclear leukocytes
NEUT = Mature polymorphonuclear leukocytes
LYM = Lymphocytes
MONO = Monocytes
EOS = Eosinophiles

Means by Treatment (June 1972)

	CA	R	I	SO_x	$R + SO_x$	$I + SO_x$	$NO_L +$ NO_{2H}	$NO_H +$ NO_{2L}
TP	6.412	6.627	6.627	6.520	6.690	6.673	6.730	6.560
RBC	7.495	7.595	7.269	7.494	7.546	7.220	7.095	7.143
HG	16.709	16.527	16.575	16.113	16.300	15.523	16.175	15.645
PCV	49.588	49.364	48.900	48.000	49.000	46.591	47.500	47.100
MCV	65.886	65.064	67.320	64.212	64.856	64.609	67.030	65.580
MCHC	33.841	33.327	33.900	33.525	33.120	33.391	33.940	32.940
WBC	9458	10245	9670	8775	11190	10109	12590	11500
BAND	271	549	237	259	309	231	422	332
NEUT	4907	5633	5888	4523	6068	5774	6966	6602
LYM	2691	2934	2293	2731	2608	2682	2971	3217
MONO	515	571	617	623	651	619	629	652
EOS	562	557	448	463	588	297	789	416

Appendix I, cont.

Means by Treatment (Nov. 1972)

	CA	R	I	SO_x	$R + SO_x$	$I + SO_x$	$NO_L +$ NO_{2H}	$NO_H +$ NO_{2L}
TP	6.976	6.903	6.980	7.000	6.980	6.809	7.000	6.860
RBC	7.771	7.651	7.219	8.020	7.248	7.348	7.146	7.587
HG	17.544	17.500	16.675	17.594	16.525	16.341	16.550	17.250
PCV	51.588	51.200	49.200	52.313	48.100	48.545	48.800	50.700
MCV	66.482	67.080	68.170	65.400	66.400	66.118	67.840	66.970
MCHC	33.971	34.170	33.830	33.625	34.280	33.582	33.940	34.030
WBC	10117	9070	8660	10137	11060	9081	9640	9600
BAND	168	186	114	210	262	207	266	214
NEUT	5177	5422	5115	5991	6672	5850	5593	5796
LYM	2224	2305	1791	2740	2005	1829	2505	2467
MONO	730	581	634	674	678	680	600	684
EOS	650	476	419	403	681	451	587	385

Means by Treatment (May 1973)

	CA	R	I	SO_x	$R + SO_x$	$I + SO_x$	$NO_L +$ NO_{2H}	$NO_H +$ NO_{2L}
RBC	7.543	7.499	7.442	7.589	7.347	7.82	7.007	7.124
HG	17.044	16.750	17.389	17.094	16.800	15.955	15.720	16.375
PCV	50.206	49.800	50.889	50.250	50.100	47.182	47.700	48.400
MCV	66.659	66.440	68.389	66.262	68.230	66.630	68.080	67.870
MCHC	33.924	33.600	34.133	34.050	33.500	33.700	32.990	33.680
WBC	9188	8720	8344	9900	10030	9581	9920	9100
BAND	226	195	155	384	641	341	346	221
NEUT	5357	5188	5447	4318	5377	5866	5742	5004
LYM	2142	2228	1841	2309	1956	2779	2521	2910
MONO	471	364	342	436	550	523	561	505
EOS	548	575	449	620	374	393	549	364

Means by Treatment (Nov. 1973)

	CA	R	I	SO_x	$R + SO_x$	$I + SO_x$	$NO_L +$ NO_{2H}	$NO_H +$ NO_{2L}
TP	7.071	7.290	6.950	6.975	6.760	6.955	7.050	6.950
RBC	7.352	7.462	7.050	7.545	7.259	7.145	6.787	7.174
HG	16.353	16.450	15.850	16.531	16.400	15.636	16.325	16.225
PCV	48.353	48.500	47.400	48.750	48.500	46.727	46.100	47.600
MCV	65.776	65.100	67.260	64.712	68.890	65.500	68.040	66.260
MCHC	33.782	33.900	33.420	33.875	33.770	33.418	33.240	34.080
WBC	10476	9210	8460	10137	10450	9381	9350	11190
BAND	5935	308	206	258	504	464	209	413
NEUT	6591	5549	5633	6078	7116	5773	5471	7175
LYM	2423	2387	2459	2379	2107	2088	2132	2879
MONO	522	574	491	789	697	869	664	686
EOS	693	402	334	474	416	468	485	334

Appendix II

Clinical Chemistry Summary (Jan. 1970)

Mean[a]	**CA**	**R**	**I**	**SO_x**	**$R + SO_x$**	**$I + SO_x$**	**$NO_L + NO_{2H}$**	**$NO_H + NO_{2L}$**
Calcium	9.77	9.58	10.06	9.95	9.98	9.81	9.95	9.56
Inorganic phosphorus	4.96	4.96	4.85	5.06	5.01	4.90	4.93	4.96
Glucose	79.2	76.8	76.0	83.2	82.0	74.5	82.7	75.9
Urea nitrogen	19.9	18.9	18.1	20.7	19.2	19.0	19.6	18.1
Uric acid	0.721	0.609	0.770	0.700	0.700	0.918	0.664	0.591
Cholesterol	186	182	199	199	168	182	174	177
Total protein	6.68	6.62	6.63	6.78	6.71	6.66	6.82	6.32
Albumin	1.76	1.54	1.77	1.76	1.76	1.77	1.76	1.68
Globulin	4.93	5.08	4.86	5.02	4.95	4.88	5.06	4.64
A/G ratio	0.347	0.309	0.350	0.335	0.320	0.345	0.336	0.336
Total bilirubin	0.195	0.155	0.170	0.218	0.190	0.182	0.173	0.155
Alkaline phosphatase	43.7	38.8	28.7	35.3	39.8	31.9	40.5	40.0
Lactic dehydrogenase	107	104	133	132	103	114	92	97
S.G.O. transaminase	36.0	33.7	32.5	38.2	43.7	37.6	33.9	35.4
Protein-bound iodine	5.26	5.49	5.10	4.87	5.54	5.04	4.66	5.11
T_3 uptake	38.5	40.1	35.0	40.5	39.8	38.7	39.8	38.7
Na	143	140	144	142	145	145	141	139
K	4.90	4.89	4.96	4.94	5.19	5.01	4.94	5.00
CO_2	28.1	27.1	28.9	27.4	30.4	27.5	29.6	28.8
Cl	113	110	114	114	113	111	114	113
Triglycerides	95	78	88	109	84	96	79	98

[a] Calcium — mg/100 ml
Inorganic phosphorus — mg/100 ml
Glucose — mg/100 ml
Urea nitrogen — mg/100 ml
Uric acid — mg/100 ml
Cholesterol — mg/100 ml
Total protein — g/100 ml
Albumin — g/100 ml
Globulin — g/100 ml
Total bilirubin — mg/100 ml
Alkaline phosphatase — milli-international units/ml
Lactic dehydrogenase — milli-international units/ml
S.G.O. transaminase — milli-international units/ml
Protein-bound iodine — μg/100 ml
T_3 — percent
Na — meq
K — meq
CO_2 — meq
Cl — meq
Triglycerides — mg/100 ml

Appendix II, cont.

Clinical Chemistry Summary (Sept. 1970)

Mean	CA	R	I	SO_x	$R + SO_x$	$I + SO_x$	$NO_L + NO_{2H}$	$NO_H + NO_{2L}$
Calcium	10.23	10.23	10.56	10.06	10.11	10.39	10.44	10.15
Inorganic phosphorus	4.79	4.90	5.26	4.91	5.29	4.73	4.77	4.99
Glucose	103	95	103	104	98	100	103	115
Urea nitrogen	16.78	17.91	15.20	17.00	17.20	14.18	14.36	21.0
Uric acid	0.556	0.518	0.49	0.512	0.620	0.455	0.518	0.600
Cholesterol	73	95	76	166	165	180	146	178
Total protein	6.83	6.74	6.65	6.49	6.64	6.39	6.97	7.00
Albumin	1.46	1.49	1.49	1.34	1.51	1.40	1.34	1.56
Globulin	5.37	5.25	5.16	5.15	5.23	4.99	5.64	5.45
A/G ratio	0.278	0.278	0.291	0.265	0.292	0.285	0.248	0.292
Total bilirubin	0.306	0.225	0.210	0.263	0.280	0.277	0.255	0.255
Alkaline phosphatase	50.6	50.0	42.0	49.4	46.5	41.4	46.8	45.5
Lactic dehydrogenase	83.1	68.0	75.0	86.3	55.5	45.0	55.5	73.2
S.G.O. transaminase	37.5	33.5	33.5	50.0	32.0	31.8	34.5	50.9
Protein-bound iodine	6.18	5.88	6.42	6.11	6.22	5.09	5.16	5.86
T_3 uptake	41.1	41.5	43.5	41.9	42.0	42.0	42.1	41.5
Na	146	146	145	146	146	146	145	145
K	4.88	5.04	5.20	4.98	4.92	4.86	4.68	5.03
CO_2	37.2	36.0	38.0	37.0	37.2	35.9	36.5	38.4
Cl	114	117	118	116	116	116	117	117
Triglycerides	50.9	68.8	76.1	57.3	79.2	58.5	52.7	96.9

Appendix II, cont.

Clinical Chemistry Summary (April 1971)

Mean	CA	R	I	SO_x	$R + SO_x$	$I + SO_x$	NO_L + NO_{2H}	NO_H + NO_{2L}
Calcium	10.33	10.49	10.35	10.13	10.49	10.53	10.25	10.50
Inorganic phosphorus	3.57	3.74	3.81	3.74	3.61	3.94	3.74	3.81
Glucose	78.3	76.4	78.5	81.9	76.0	76.8	75.9	74.5
Urea nitrogen	10.61	9.09	9.80	10.25	10.20	9.64	10.82	11.10
Uric acid	0.867	0.573	0.680	0.775	0.880	0.995	0.782	0.860
Cholesterol	179	212	165	185	175	178	156	160
Total protein	6.72	6.76	6.36	6.59	6.86	6.59	6.79	6.89
Albumin	1.544	1.518	1.480	1.438	1.530	1.527	1.500	1.550
Globulin	5.17	5.24	4.88	5.15	5.33	5.06	5.29	5.34
A/G ratio	0.267	0.264	0.250	0.250	0.250	0.264	0.264	0.250
Total bilirubin	0.272	0.282	0.260	0.214	0.280	0.318	0.373	0.280
Alkaline phosphatase	50.6	50.9	43.2	44.4	52.7	40.9	56.8	50.8
Lactic dehydrogenase	113	120	111	108	128	123	149	126
S.G.O. transaminase	37.2	34.7	35.4	34.0	40.0	36.1	39.5	40.8
Protein-bound iodine	5.41	5.21	4.79	6.25	5.52	5.01	5.09	5.48
T_3 uptake	41.4	41.5	41.9	41.4	41.7	40.4	41.5	41.7
Na	145	145	146	146	146	147	146	145
K	4.81	5.00	4.89	4.78	4.84	4.83	5.05	4.78
CO_2	37.9	38.6	38.8	38.9	38.0	38.5	38.4	37.8
Cl	104	103	106	105	105	106	105	104
Triglycerides	74.9	86.1	63.0	75.5	81.2	69.3	70.3	65.0

Appendix II, cont.

Clinical Chemistry Summary (Nov. 1973)

Mean	CA	R	I	SO_x	$R + SO_x$	$I + SO_x$	$NO_L +$ NO_{2H}	$NO_H +$ NO_{2L}
Calcium	9.20	9.00	8.96	9.39	8.92	9.26	9.040	9.010
Inorganic phosphorus	3.41	3.86	3.43	3.83	3.68	3.80	3.30	3.72
Glucose	85	91	82	82	79	83	86	81
Urea nitrogen	12.18	10.70	10.67	10.38	11.10	12.09	11.10	12.00
Uric acid	0.512	0.450	0.511	0.475	0.450	0.518	0.440	0.460
Cholesterol	211	245	189	188	183	216	189	198
Total protein	6.62	6.63	6.41	6.55	6.49	6.54	6.60	6.79
Albumin	2.72	2.86	2.68	2.66	2.70	2.66	2.59	2.71
Globulin	3.90	3.77	3.73	3.89	3.79	3.87	4.01	4.08
A/G ratio	0.712	0.850	0.744	0.687	0.710	0.709	0.650	0.700
Total bilirubin	0.212	0.320	0.278	0.200	0.300	0.245	0.270	0.430
Alkaline phosphatase	60.3	69.0	41.7	49.4	55.5	43.6	47.5	84.5
Lactic dehydrogenase	227	304	259	209	282	232	190	243
S.G.O. transaminase	37.4	45.0	47.2	35.0	46.5	45.9	44.5	46.5
T_3 uptake	46.6	44.8	47.6	47.1	44.9	42.9	45.8	42.2
Na	146	145	147	146	147	146	145	147
K	4.82	4.88	4.96	4.81	5.12	5.06	4.85	4.93
Cl	110	109	108	110	110	108	111	112

Asilomar Conference Discussion

Hueter: This is like going back to a class reunion, being here with a lot of the people who are here today, and I would like to make a few thank yous if that is appropriate, particularly to two gentlemen who are no longer with us, Mr. Eugene Krakow and Charlie Punty, who were heads of the laboratory at the time this study was started, and were instrumental in getting support for this chronic study from our headquarters people. At that time, we were with the U.S. Department of Health, Education, and Welfare. Many of you have been involved with chronic studies in the past, and maybe some of you haven't, but I think we learned a lot in conducting this. As Dr. Stara said earlier, we would do things a lot differently now if we did it over again, but under the circumstances I think the study was done quite well. Mr. Hinners and Joe Burkart, who were our engineers, put together an excellent facility that I don't think exists anywhere else in the world. Mr. Krider and Malanchuk had the chore of doing these routine monitorings of the atmospheres for many, many years. It's very difficult to find people, scientists who would dedicate that much of their lives to this kind of a study. Most people want a lot more visibility than one gets from this type of work. Of course, Ken Busch pointed out the part that statistics played and we were fortunate in having him and his staff. I think the biologists involved should be complimented for being willing to cooperate the way they did with the statisticians when the study was started, so at least we could get as much out of it as possible under the circumstances. Drs. Lewis and Campbell and many other people that were associated with the study devoted a lot of their time to something with an unknown end product. I might point out, the question was raised before as to why the study was stopped. The study cost us, I believe, about $250,000 a year to run. This included staff time and supplies, that's to say nothing of what the facility cost to install, which I think was 1½ million dollars. Our budget at that time was somewhere in the order of $900,000, so you can see we were spending over 25% of our laboratory budget on this facility, and consequently we were getting shot at every year when it was program planning time and budget time. What have we found out, what do you see, have you seen anything, stop the study. So, it was a constant battle from that point of view. The reason the study was stopped was in part that kind of pressure and in part that at that particular time, the facility was being considered for removal from Cincinnati and relocation in RTP in North Carolina. This meant a tremendous disruption in the facility and the staff, so serious questions were then being raised as to continuing the study. Compromises were then made, about where the animals would be maintained. I'm not sure what my opinion was at that time, but I'm certainly glad that whatever it was, it occurred the way it did. I might point out before this study was started there were some very serious and detailed studies done with the chamber system. The dogs were held in runs for a period of time to check them for their general health. They were then put in the chambers for a period of almost 2 months to condition them to that type of living facility before they were exposed to any atmospheres at all. The chambers themselves before the dogs were even placed in them were characterized over a period of 6 weeks. We looked at what concentration of pollutant was coming in at the top, what concentration was going out the bottom. What were the chamber losses, in other words. We had different cage configurations in the chambers, to look at that effect on the losses across the chamber of things like ozone and NO_x. We then had different cage configurations in animal loadings, to look at the effects of the animals on losses across the chamber. So that when we finally did start the study, we could have a minimization at least of loss of what the animals on the bottom were receiving relative to the animals on the top. So a lot of work went into this before the study was even started. The reason these atmospheres were selected, of course, is pretty obvious. We were interested at that time in auto exhaust, catalytic oxidation catalysts were not even thought of at that time, and we were also interested in NO_x as an individual pollutant because it of course is a source of pollution from point sources as well as mobile sources. And with a limited number of chambers, that was the reason we picked those eight atmospheres, which include the clean air. We had done another study prior to this with auto exhaust using smaller chambers and using all rodents, hamsters, rats and mice, in which we attempted on an engineering basis to expose animals to four different levels of auto exhaust, irradiated and nonirradiated, and cycling it diurnally over a period of 24 hours to simulate the type of thing that happens, for example, in Los Angeles. This was extremely difficult to do from an engineering point of view. There was a lot of overlap between the various levels that we attempted to reproduce. That's why we decided on trying to maintain the exposure atmospheres at a constant level over a period of 16 hours per day, 7 days a week. This is not realistic from the point of view of city atmospheres, it was realistic from the point of view of cutting down the variability of the study. We did, as Ken Busch pointed out, control about as many variables as we possibly could. The windows in the room were blocked off so all animals had artificial light. The visitors were not allowed to run in and out looking at the dogs, so we controlled the amount of activity in the room. The chambers were operated under a half-inch of negative pressure so that there was no leakage from the chamber into the room, and the room in which the chambers were located itself also had its own clean air supply, its own CBR filters, its own air conditioning humidity capability, so when the doors were opened the animals weren't exposed to a lot of pollutants which might have been coming in from the city atmosphere itself. It was also necessary in order to maintain the temperature in chambers since these were all stainless steel glass chambers. The dogs were, I believe, all within 2 weeks of the same age when the study began.

Albert: Were these dogs abnormally healthy?

Hueter: No, I wouldn't say so.

Albert: You didn't lose a single one from disease over 9 years?

Hueter: They weren't exposed to very much where they should be getting disease.

Thurlbeck: Three died from disease.

Orthoefer: These were from inguinal hernias. These dogs had a very high incidence of inguinal hernias; the hernia had strangulated, and the animal died.

MacEwen: Dogs on long-term experiments tend to get healthier with time because they are not exposed to outside environmental influences and they don't get infections because they are not exposed to other dogs. They see only the others in their chamber so they become highly stabilized. In my experience, in our laboratory at Wright-Patterson, we get much narrower ranges of normal values within the experimental groups maintained in chambers for long periods of time where temperature, humidity and feeding times are relatively constant.

Stara: This is the end of this session. Thank you, gentlemen, for a fruitful discussion.

4. COLLAGEN AND PROLYL HYDROXYLASE LEVELS IN LUNGS IN DOGS EXPOSED TO AUTOMOBILE EXHAUST AND OTHER NOXIOUS GAS MIXTURES

R.S. Bhatnagar

Summary

The effect of prolonged exposure to a variety of noxious environments on lung connective tissues was examined in beagle dogs. The animals were exposed to raw and photochemically reacted automobile exhausts, oxides of nitrogen and sulfur, alone and in combination with exhausts for over 5 years, following which they were placed in ambient air for 2½ to 3 years. No significant differences were seen in the concentrations of collagen in exposed and unexposed animals. Significant differences were seen, however, in prolyl hydroxylase levels between control lungs and lungs exposed to irradiated exhausts (I), a high concentration of nitric oxide plus a low concentration of nitrogen dioxide ($NO_H + NO_{2L}$), and irradiated exhaust plus sulfur oxides ($I + SO_x$). Exposure to low nitric oxide plus high nitrogen dioxide ($NO_L + NO_{2H}$), to oxides of sulfur (SO_x), to oxides of sulfur plus nonirradiated exhaust ($R + SO_x$) and to nonirradiated exhaust alone (R) produced smaller differences in prolyl hydroxylase levels.

Introduction

Exposure of lungs to a variety of noxious gaseous and particulate chemical agents results in tissue damage and impairment of pulmonary function. These changes in lungs are characterized by connective tissue growth and distortion of lung architecture (1,2). Although only morphological evidence has been available, it has been assumed that these changes involve increased deposition of collagen, the major protein component of connective tissues. Despite mounting concern over respiratory disorders directly related to automobile exhaust emissions, few systematic investigations have been made of the biochemical effects of the toxic agents contained in internal combustion engine exhausts and resulting from the interaction of these exhausts with the environment. The EPA-UC Davis study on the effect on pulmonary function of nonirradiated and irradiated automobile exhausts and mixtures of oxides of sulfur or nitrogen has been a major effort in elucidating this question. The major components of this study were pulmonary function analyses and morphological studies. Analysis of data from these investigations should yield invaluable information concerning the health effects of automobile exhausts. The studies, however, originally did not include biochemical examination of pulmonary connective tissues. Biochemical studies were initiated during the sacrifice phase of the EPA-UC Davis project.

The objective of this study was to analyze the collagen content and the ability to synthesize collagen in the lungs of dogs exposed to a variety of noxious gaseous environments for over 5 years and subsequently maintained in ambient air for over 2 years.

Materials and Methods

Our studies were carried out using frozen samples of parenchymatous tissue provided by Dr. J. Orthoefer. Because of difficulties involved in obtaining and transporting viable tissues immediately after sacrifice, it was not possible to assay cellular protein and collagen synthesis.

It was only possible to measure the collagen content as a fraction of the total protein content of the tissue. Changes in the collagen content of impaired lungs should be reflected in altered ratios of collagen to total protein content.

Since it was not possible to assay directly for collagen synthesis, we measured the levels of prolyl hydroxylase, a key enzyme involved in the synthesis of collagen. Prolyl hydroxylase levels generally reflect the potential of a tissue to synthesize collagen and are elevated under conditions of enhanced collagen synthesis.

Details of the exposure of the beagle dogs to the various environments have been discussed in Chapter 1. The tissues used in the present investigation were packed in dry ice and shipped from the University of California at Davis to our laboratory at the University of California, San Francisco. The tissues were allowed to thaw at cold room temperatures and were rinsed in chilled distilled water to remove extraneous protein and other adhering materials. Portions of the tissues were homogenized in 0.1 M TRIS HCl, 0.1 M KCl, at pH 7.4, and the homogenates were centrifuged at 15,000 x G to obtain a supernatant which was used as the crude prolyl hydroxylase preparation. Small portions of the tissues were hydrolyzed in 6 N HCl for the assay of protein and collagen content.

Prolyl hydroxylase activity was assayed using a substrate labeled with (3,4-$^3H_2$)-proline. The substrate was prepared by incubating 10-day embryonic chick cartilage explants with the labeled proline in the presence of 1.0 mM *a,a'* bipyridyl (3). Prolyl hydroxylase reaction was carried out as described by Bhatnagar *et al.* (3) and Rapaka *et al.* (4). During the reaction, tritium is released in stoichiometric proportions to proline hydroxylated. ^{3}HH0 is distilled under vacuum, and radioactivity in the collected ^{3}HH0 is used as a measure of enzyme activity. In these experiments the activity of prolyl hydroxylase is expressed as dpm ^{3}HH0 released per mg of protein in the lung homogenate.

Protein and collagen contents were determined in hydrolyzates of the tissues. The tissues were hydrolyzed in 6.0 N HCl, under N_2, for 20 hours. The hydrolyzates were decolored with activated charcoal and evaporated to dryness in a rotatory evaporator, under vacuum. The residues were dissolved in 5 ml distilled water, and appropriate dilutions and aliquots were used for the assays.

Protein content of the hydrolyzates was measured using the method of Rosen (5) by assaying for the total ninhydrin reactive materials, using leucine standards. These data are expressed in terms of leucine equivalents. Collagen was assayed on the basis of hydroxyproline content using Woessner's method (6). Hydroxyproline is present only in collagen. Therefore a specific measurement for hydroxyproline gives a measure of collagen content. Since the objective in these studies was to determine whether there was any change in the tissue content of collagen, the ratio of collagen to total protein was considered. This is expressed in terms of the ratio of hydroxyproline to total ninhydrin reactive material (in leucine equivalents).

Results

Tissue concentration of hydroxyproline was used as an index of collagen concentration, and total ninhydrin reactive material was used as an index of total protein content. Thus, the experiment was designed to detect possible changes in collagen-proline ratios. No significant differences were found in the quotient hydroxyproline/total ninhydrin reactive material (Tables 1 and 2); nor did the preliminary histologic and other morphological studies on these tissues indicate major differences in the collagen content of exposed tissues.

As described, the relative levels of prolyl hydroxylase were determined in the tissues (Tables 3 and 4). Although no significant differences were observed in the collagen protein ratios between tissues derived from dogs receiving different treatments, significant differences were seen in the levels of prolyl hydroxylase. Lungs from animals exposed to irradiated exhaust had the highest levels of prolyl hydroxylase. The nitrogen oxide environments and irradiated exhaust in combination with sulfur dioxides also resulted in an increase in prolyl hydroxylase activity. Sulfur oxides caused only minimal increases in the enzyme level and raw exhaust alone and in combination with sulfur oxides did not significantly alter the enzyme levels.

Discussion

Our data indicate that there were no significant differences in the tissue concentrations of collagen between the control and exposed animals. Crystal and colleagues (7,8) also failed to detect significantly altered collagen concentrations in the lungs of patients with severe symptoms of fibrosis. A possible explanation for this has been provided by recent studies on the effect of short- and long-term exposure of rat lungs to ozone (9,10) and to nitrogen oxides (11). In these studies, the rate of collagen synthesis in the lungs of animals exposed to the toxic environment was significantly increased during the initial exposure. The initial spurt in collagen synthesis was followed by increased synthesis of all proteins, until no difference could be detected between the rates of collagen synthesis and of overall protein synthesis, indicating that all tissue protein components were deposited at the same rates. After prolonged exposure

Table 1. Analysis of Variance for Hydroxyproline/Ninhydrin (X 10^4)[a,b]

Source of variation	df	MS	F	P
Treatment	7	3972	1.261	0.295
Error	39	3151		
Total	46			

[a]Hydroxyproline/Ninhydrin = collagen (specific activity)
[b]Orthoefer *et al.* (27)
Key: df = degrees of freedom
MS = mean square
F = "f" test
P = significance

Table 2. Mean Hydroxyproline/Ninhydrin (X 10^{-4}) by Treatment and the Results of Duncan's Multiple Range Test[a]

I	R	NO_L + NO_{2H}	NO_H + NO_{2L}	CA	R + SO_x	I + SO_x	SO_x
425	388	381	370	363	344	344	341

[a]Orthoefer *et al.* (27)

Table 3. Analysis of Variance for Prolyl Hydroxylase[a,b]

Source of variation	df	MS	F	P
Treatment	7	7324411	13.480	0.000
Error	39	543347		
Total	46			

[a]Orthoefer *et al.* (27)
[b]See Table 1 for key.

Table 4. Mean Prolyl Hydroxylase (dpm/mg) by Treatment and the Result of Duncan's Multiple Range Test (a = 0.05)[a]

I	NO_H + NO_{2L}	I + SO_x	NO_L + NO_{2H}	SO_x	R	R + SO_x	CA
5351	3339	2970	2958	2030	1931	1680	1622

[a]Orthoefer *et al.* (27)

to ozone, there were no detectable differences in collagen concentrations between control and exposed animals (10). These studies also revealed a marked capacity of the lung to repair itself after the fibrogenic stimulus had been removed (9).

Although biochemical data are not available for the initial phases of the dog study, it is possible that dog lung connective tissue underwent a course of biochemical changes similar to those observed in the rat studies. Thus it is conceivable that during the initial exposure period, the chronically exposed beagle lungs underwent a concomitant increase in collagen and non-collagenous proteins leading to increased tissue mass without chemically discernible alterations in collagen concentrations. During the chronic exposure and subsequent maintenance in "clean" air, any initial alterations in the connective tissue would have been obliterated, as seen in the rat studies (9-11). This explanation does not preclude any pathological alterations in pulmonary mechanics and other physiological parameters since these properties are dependent on the macro- and micro-architecture of the alveolar wall, which would undergo drastic changes as a result of both increased collagen and non-collagenous protein deposition. Furthermore, any repair processes involving local deposition of scar tissue and increased turnover of pre-existing connective tissue components would also lead to altered mechanics without quantitative alterations in the tissue protein and collagen contents.

The limited nature of these studies did not permit evaluation of qualitative differences in the collagen contents of control and exposed animals. Collagen comprises a family of genetically related proteins. Several different polymorphs of collagen have been described. Many of these collagen species are tissue-specific. Type I collagen, the most abundant collagen species in the body, is present in most tissues and organs, and is also a major component of pulmonary connective tissue, being present in the large bronchii and blood vessels. It is synthesized by the parenchyma (12, 13). Lung mesenchymal cells in culture synthesize type I collagen (14), as do parenchymal cells of apparent epithelial origin (7, 15). Many cells of non-mesenchymal origin also synthesize collagen (16, 17), hence it may be presumed that some collagen may be synthesized in the lungs by non-mesenchymal cells. Type II collagen, a component of cartilage, is present in trachea and bronchii as well as in other tissues (12). It may be synthesized by chondrocytes in tracheal and bronchial cartilage. Type III collagen is present in the lungs, but its structural location is not known. It is synthesized by lung mesenchymal cells in culture (14). Type I and II collagens are also synthesized by lung epithelial cells in culture (7, 15). Type IV collagen, the principal collagen component of basement membrane, is associated with alveolar septa. It may be synthesized by lung epithelial and endothelial cells.

Pulmonary parenchyma is a complex tissue with considerable variation from site to site. Different parts of the parenchyma may contain different types as well as different amounts of collagen. Variability may also be manifested in the collagen-associated structural proteins. Measurements of collagen in the parenchyma tend to average out these variations, and thus may mask experimentally induced effects. Changes in collagen in a small but crucially important component, or destruction of collagen in one component with a commensurate increase in another, may elude detection by chemical measurements, since overall collagen content may be unaltered. Such changes may in some cases be identified by morphological examinations. For example, the destruction of basement membrane collagen, which does not have a clearly defined fibrous structure, and its subsequent replacement with scar tissue collagen, which is organized as loose fibers, may give the appearance of increased collagen deposition even though the net concentration of collagen may have remained unaltered.

Although it was not possible to measure directly the ability of lung explants to synthesize collagen *in vitro*, it was possible to assay the tissues for their prolyl hydroxylase content. Prolyl hydroxylase is a critical enzyme in the pathway of collagen synthesis, hence its presence in a tissue usually denotes a potential for collagen synthesis. In lungs (9, 10, 19) and other tissues (20-22) engaged in enhanced collagen synthesis, the levels of prolyl hydroxylase are elevated. Our study on prolyl hydroxylase levels revealed a marked difference between control tissues and tissues that were exposed to irradiated exhausts (I), $NO_H + NO_{2L}$, I + SO_x, and $NO_L + NO_{2H}$. Exposure to SO_x and to raw exhaust (R) also caused increased enzyme levels although the difference was not as large. Surprisingly, exposure to R + SO_x did not elicit any change. In the absence of data on the rates of collagen and protein synthesis in the explants, it is not possible to explain the significance of these findings. It may be speculated that the tissues with the higher levels of prolyl hydroxylase were somehow more actively turning over collagen and were in a dynamic state of repair and regeneration.

In order to obtain a clear understanding of the reasons for the differences in prolyl hydroxylase content, it would be necessary to obtain correlations between the prolyl hydroxylase levels and various mechanical and physiological parameters. Altered mechanical characteristics accruing from the altered structure of the alveolar septa may result in a continuing injury situation and a continual reparative response, leading to an elevated collagen turnover rate. Higher collagen turnover rates may reflect a situation where both synthesis and breakdown proceed at equal but elevated rates. This would explain increased prolyl hydroxylase levels without any apparent changes in the net composition of the tissue.

An interesting aspect of these observations is that the highest prolyl hydroxylase levels were seen in tissues most likely to be exposed to ozone since both irradiated exhaust and oxides of nitrogen can lead to the formation of ozone. Ozone and other oxidant gases generate superoxide radicals in biological systems (23). Our studies have indicated that superoxide radicals are an important reactive species in the prolyl hydroxylase reaction (24). Our recent studies suggest that the superoxide radicals induce collagen synthesis in a variety of cells including fibroblasts of pulmonary origin in cultures (25, 26). These studies showed that superoxide also induces prolyl hydroxylase in these cells. The implication of these observations is that the induction of pulmonary fibrogenesis may involve the generation of superoxide radicals. Generation of superoxide by oxidizing gases may thus be a part of their fibrogenic effect on lungs.

Our studies raise several important questions:

1. What is the role of turnover rates of connective tissue macromolecules in pulmonary fibrosis?

2. Are the differences in the mechanical and physiological characteristics of control and exposed lungs attributable to morphological alterations involving *all components* rather than connective tissue alone?

3. What type of biochemical changes occurred immediately after the initial exposure?

4. Since the experiment lasted several years, could the effects of aging be separated from the pollution effects?

5. What kinds of changes occurred in the macromolecular content of the tissues? Alteration in the types of collagen, elastin, etc., would lead to altered mechanical properties.

In concluding this report, it is suggested that serious consideration be given to a thorough examination of connective tissue turnover and the chemical and physicochemical nature of its components throughout the course of this type of a study. Since the mechanical and physiological functions of the lungs are entirely dependent on the connective tissue component of lungs, this point cannot be overemphasized.

References

1. Liebow, A.A. 1975. Definition and classification of interstitial pneumonias in human pathology. Prog. Resp. Res. 8: 1.
2. Spencer, H. 1975. Pathogenesis of interstitial fibrosis of the lung. Prog. Resp. Res. 8: 33.
3. Bhatnagar, R.S., R.S. Rapaka, T.Z. Liu, and S.M. Wolfe. 1972. Hydralazine-induced disturbances in collagen biosynthesis. Biochim. Biophys. Acta 271: 125.
4. Rapaka, R.S., K.R. Sorenson, S.D. Lee, and R.S. Bhatnagar. 1976. Inhibition of hydroxyproline synthesis by palladium ions. Biochim. Biophys. Acta 429: 63.
5. Rosen, H. 1957. A modified ninhydrin chlorometric analysis for amino acids. Arch. Biochem. Biophys. 67: 10.
6. Woessner, J.F., Jr. 1961. The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. Arch. Biochem. Biophys.•43: 440.
7. Hance, A.J., A.L. Horowitz, M.J. Cowan, N.A. Elson, J.F. Collins, R.S. Bienkowsi, K.H. Bradley, S.D. McConnel-Breul, W.M. Wagner, and R.G. Crystal. 1976. Biochemical approaches to the investigation of fibrotic lung disease. Chest Suppl. 69: 257.
8. Fulmer, J.D., and R.G. Crystal. 1976. P. 419 *in* R.G. Crystal, ed. The biochemical basis of pulmonary function. Marcel-Dekker, New York.

9. Hussain, M.Z., C.E. Cross, M.G. Mustafa, and R.S. Bhatnagar. 1976. Ozone-induced pulmonary fibrosis: synthesis of collagen in lungs of rats exposed to ozone. Life Sciences 18: 897.
10. Cross, C.E., K. Reddy, M.Z. Hussain, L.W. Schwartz, M.G. Mustafa, and D.L. Dungworth. 1972. Lung adaptation to low levels of ozone on long-term exposure. Am. Rev. Resp. Dis. 113: 102.
11. Hacker, A. 1975. Effects of nitrogen dioxide on collagen metabolism. Ph.D. thesis, U.C.L.A.
12. Bradley, K.H., S.D. McConnell-Breul, and R.G. Crystal. 1974. Lung collagen composition and synthesis. J. Biol. Chem. 249: 2674.
13. Bradley, K.H., S.D. McConnell-Breul, and R.G. Crystal. 1974. Lung collagen heterogeneity. Proc. Natl. Acad. Sci. 71: 2828.
14. Hance, A.J., K. Bradley, and R.G. Crystal. Lung collagen heterogeneity. II. Synthesis of Type I and Type III collagen by rabbit and human lung cells in culture. J. Clin. Invest. 57: 102-111.
15. Elson, N., K. Bradley, A. Hance, A. Kniazeff, S. Breul, A. Horowitz, and R.G. Crystal. 1975. Synthesis of collagen in cultured lung cells. Clin. Res. 23: 346A.
16. Green, H., and B. Goldberg. 1965. Synthesis of collagen by mammalian cell lines of fibroblastic and non-fibroblastic origin. Proc. Natl. Acad. Sci. 53: 1360.
17. Langness, U., and S. Udenfriend. 1974. Collagen biosynthesis in non-fibroblastic cell lines. Proc. Natl. Acad. Sci. 71: 50.
18. Grant, M.E., N.A. Kefalides, and D.J. Prockop. 1972. The biosynthesis of basement membrane collagen in embryonic chickens. J. Biol. Chem. 247: 3539.
19. Halme, J., J. Uitto, K. Kahanpaa, P. Karkunen, and S. Lindy. 1970. Protocollagen proline hydroxylase activity in experimental pulmonary fibrosis of rats. J. Lab. Clin. Med. 75: 535.
20. Takenchi, T., K.I. Kivirikko, and D.J. Prockop. 1967. Increased protocollagen hydroxylase activity in the livers of rats with hepatic fibrosis. Biochem. Biophys. Res. Comm. 28: 940.
21. Uitto, J., J. Halme, M. Hannuksela, P. Peltokallio, and K.I. Kivirikko. 1969. Protocollagen proline hydroxylase activity in the skin of normal human subjects and of patients with scleroderma. Scand. J. Clin. Lab. Invest. 23: 241.
22. Mussini, E., J.J. Hutton, Jr. and S. Udenfriend. 1967. Collagen proline hydroxylase in wound healing, granuloma formation, scurvy, and growth. Science 157: 927.
23. Fridovich, I. 1972. Superoxide radical and superoxide dismutase. Accts. Chem. Res. 5: 321.
24. Bhatnagar, R.S., and T.Z. Liu. 1972. Evidence for free radical involvement in the hydroxylation of proline: inhibition by nitroblue tetrazolium. FEBS. Lett. 26: 32.
25. Hussain, M.Z., J. McManus, J.J. Ludoweig, and R.S. Bhatnagar. 1976. Binding of α-ketoglutarate to prolyl hydroxylase. Fed. Proc. 35: 1570.
26. Bhatnagar, R.S., J. McManus, and M.Z. Hussain. 1976. 10th International Congress Biochem. Hamburg.
27. Orthoefer, J.G., R.S. Bhatnagar, A. Rahman, Y.Y. Yang, S.D. Lee, and J.F. Stara. 1976. Collagen and prolyl hydroxylase levels in lungs of beagles exposed to air pollutants. Environ. Res. 12: 299.

Asilomar Conference Discussion

Bhatnagar: The data that we have provided were based on all the tissues we received and I had no idea which animals were which. Mr. Yang did the statistical analysis.

Orthoefer: I think it has to do with the duplicate samples.

Bhatnagar: That's right.

Albert: Could you give us a rough idea of how many animals there are, for instance, in the irradiated group?

Bhatnagar: I'm very sorry, I don't have those figures. I asked for summarized data, and that's what I have. For the total collagen content as well as the prolyl hydroxylase, our analysis, based on simple statistics, matched the analysis with a limited number of samples. We did analyses of all the samples we had, and eliminated some of the samples because they were too far out of the range.

Kleinerman: Did you know the identity of each sample you analyzed?

Bhatnagar: No, we had no idea. They were done totally blind. In fact, for the longest time I had no idea of what these results meant although we had all the data. It took an awful lot of time to generate data on 86 animals and doing each one in triplicate or quadriplicate and so forth. We did these analyses on two different occasions. The result of all that was that, even after doing all this work, we had no idea what we had done, because we did not have the code. I was unable to get the code.

Stara: The blind on analytical work was done on purpose after discussion to see if there would be some valuable data coming. As far as the 47 dogs, I will send the information within weeks to each of you.

Lewis: Dr. Hueter and I, and others in the laboratory, were interested in this type of biochemical reaction 10 years ago or more, and at the time we analyzed tissues from animals in the first auto exhaust studies and got the same kind of results you did and so did many other colleagues in the field. That's 10 years ago. The hypothesis was at that time that it's not an increase in the content of collagen or elastin, but it's a physical/chemical rearrangement or cross-linking to hydrogen bonding, amino end groupings of lysine, other amino acids, etc. I'm quite surprised that you looked at it in this fashion rather than from a physical/chemical fashion.

Bhatnagar: It would be very nice to look at it that way, but from the amount of tissue that we got, there wasn't very much we could do.

McLees: I don't know how you look at it in a physical/chemical fashion. It's very tough. Most of that work has been done, say with something like tendon, of that nature, and that's a very indirect kind of thing. It is possible to look at cross-links reasonably directly with this tissue and it could be done probably now with the tissue using chlorohydride reduction, that is, introducing tritiated tritium into the reducible cross-links and subjecting that to alkaline hydrolysis and passing it over a specific ion exchange cross-link analyzer. The trouble with that is that these animals, I take it, are fairly old, as far as animals go, and most of the reducible cross-links are physiologically reduced as part of aging, so I'm not sure what you would get from this even with that, but that's do-able and you could at least probably tell if the cross-links are more and more abnormal.

Lewis: Is the hypothesis of increased cross-linking with aging of collagen and elastin the current one accepted, or are there other more tenable hypotheses in acceptance today?

McLees: I don't know how to answer that; it depends on who's making the hypothesis. I would say, I guess my thought is that there are not enough data at the molecular level to allow you to choose between one hypothesis and another.

Bhatnagar: One of the problems with lung collagen is that it is comparatively unstable. It does turn over quite rapidly, much more rapidly than some other collagens in the body. So over this period of time, we had some new collagens synthesized. If there were any difference in cross-linking and so forth, introduced during the exposure part of the study, you probably would not expect to see them, at the end, anyway.

Lewis: We had an extremely difficult time demonstrating increased collagen deposition in animals exposed to material like coal dust, much less trying to demonstrate it with this type of exposure. I'm talking about 10 milligrams per cubic meter loads, like that. Dr. Lee, have you any experience with collagen analysis in your work?

Dr. Lee: No.

Question: Are there any more questions?

Tyler: I would like to ask one, please. Ninhydrin reaction. It's been a long time since I thought about it, but plasma proteins react also, don't they? So that if you corrected these essentially for the hematocrit for the amount of blood left in the tissues, which would relate to the protein left in the tissues in the bloodstream, perhaps then your collagen index now is structural collagen against total protein, and the total protein is going to vary with how much blood was left in the lung at the time the animal was killed. So if you correct for the amount of blood left in the lung at that time it was killed by hemoglobin concentrations, much the way Staub does, then you would perhaps make more sense out of the collagen index because it would be a true index instead of an index which is influenced by the amount of blood present.

Bhatnagar: We'll talk about that, but I would think it is corroborated by the two or three other studies where they did not find any changes in the collagen concentrations, as in studies from Dr. Crystal's lab at the Heart and Lung Institute and from the studies that Dr. Hussain did, just looking at the rates of collagen synthesis. They also seem to suggest that there are possibly mechanisms which will bring the concentrations of collagens down to normal. I'm talking about the concentrations now. There may be qualitative changes in collagens which could not be seen in other studies.

Albert: Do you know any conditions of the lung where collagen turnover has been accelerated?

Bhatnagar: Perhaps Dr. McLees can answer that.

McLees: Not that I know of, but after you remove the lung, it seems to lay down more collagen at a very rapid rate.

McLees: Well, that's true, but everything else is turned on with that. The whole lung grows after you do an embinectomy and so it's kind of everything going, but even so, the turnover, when we look at, say, people with idiopathic fibrotic lung disease, using labeled collagen and lung exponents, their rate of synthesis of collagen is normal compared to controls in biopsy specimens. These are people with a classic physiologic picture of lung fibrosis, classic pathologic picture, and with biochemical data to go along with that. We don't find that it's increased. I raise this question because the fraction of collagen that would turn over rapidly would be the soluble fraction probably, and our experience has been that the stuff is so insoluble in the adult lung that we get, you know, 1% or half a percent that is extractable with salt and acetic acid under those conditions. I wonder about that. I dare say I'm uncertain.... Except in these situations where everything goes up, collagen synthesis will go up along with total protein synthesis, but that's a little bit different situation.

Albert: That's not turnover, is it? That's just an increased deposition.

McLees: Right, but in the synthesis, I'm not aware of any circumstance where the turnover number you'd look at that we've done would show that. I wanted to come to this point because I think one of the important things in this study is where those biopsies of the lungs came from.

Orthoefer: They all are left diaphragmatic lobes.

McLees: One of the problems is that ratio of milligrams of collagen per milligram of dry weight will depend on how much airway tissue there is in the biopsy specimen. If you take that from the periphery of the lung, you are going to get a different ratio than if that biopsy comes up closer in to the large airways.

Orthoefer: I basically prepared all these and I clipped the major airways out, and on some of them, Dr. Bhatnagar was interested in seeing what amount of collagen was being clipped out with these, so I packaged them up separately and mailed them down to him. There should probably be some data on this. Actually, I did them all as close to the same way as possible. It is possible that a small airway was left in one and not in another, but I tried to get the major airways out of the lung.

McLees: But in answer to your question, I think the problem is tied up in this. It's a denominator problem. If you take a situation much more impressive than this in terms of pathology, where our pathologists will tell us that the lung is loaded with fibrosis, the biochemical analysis on that done in many different fashions always shows that the collagen content is normal biochemically. I think that is a problem of biochemical analysis and not knowing what to choose as a denominator. The trouble is collagen makes up so much of the lung in itself that it could be very, very difficult to pick up a change in collagen content when you're selecting a denominator such as per milligram dry weight or ninhydrin or what have you.

Orthoefer: Apparently all proteins are turned on — the total ninhydrin increases as does the collagen.

McLees: Yes, but the same thing is true of dry weight or whatever you choose. That's the problem. I guess the other question I have refers to hydroxyproline, though. That is maybe another question because in many illnesses the serum level of that protein is increased substantially. For example, in hepatitis, probably you have all-time record highs of the prolyl hydroxylase in the serum and it's been analyzed in a series of 80 different diseases and it's a very nonspecific protein elevated everywhere. That could be a problem here, if you couldn't get rid of the serum from the lung tissue. Were the lungs perfused when they were taken out?

Orthoefer: No.

Bhatnagar: The only processing of the lungs that was done in terms of cleaning them was that in my lab they were cleaned as thoroughly as they could be cleaned up.

Kleinerman: What does that mean?

Bhatnagar: What that means is that they were washed in many, many changes of buffer and homogenized in buffer for the purpose of doing the hydroxylase. Of course, we had to take as much of the tissue as possible, for the purpose of determining the hydroxyproline. What we tried to do was to take most of the insoluble material, that was not soluble in buffers that would not solubilize collagen.

Question: But you had to pull hydroxylase.

Bhatnagar: We used that wash, too, because there was obvious debris attached to these tissues and they were not clean. We tried to wash out as much of the material as possible. I don't know if when one washes these things by rinsing them thoroughly, one gets into the alveoli and gets rid of everything out of them.

Orthoefer: What do you mean by debris?

Bhatnagar: When you cut these lungs up, there was a lot of stuff, just minute chunks of tissue that were adhering to the pieces of lung; also, there were precipitated proteins, blood, and stuff that came off very easily when we washed these tissues. We tried to get rid of as much of the adhering material as we could.

McLees: I would like to take the Chairman's prerogative of asking two more questions, if I may. One of them is that I am concerned a bit about the equation of increased prolyl hydroxylase activity with increased collagen turnover, you've shown an increase in enzyme activity.

Bhatnagar: That was not an assumption, that was a speculation. I tried to explain why one might see more of it.

McLees: But it could be a denominator problem there also. Or it could be a specific activation of the enzyme that would have nothing to do with its catalysis.

Bhatnagar: Why would that occur in particular groups?

McLees: I have no idea.

Bhatnagar: I think these differences were real.

McLees: I am not arguing that the differences were real. I'm arguing about the interpretation of the difference. In other words, the fact that the enzyme activity is increased, to me means just *that*. I guess what I have more trouble with is the extrapolation from that fact to the fact that the turnover or the synthesis of the collagen is increased.

Bhatnagar: But any alternative speculations on that are quite welcome.

McLees: I offer two others. One is that your expression per unit something, ppm per milligram or something, that per is changing. The other is that one could just as easily speculate that this is an enzyme that has many cofactors, and in fact it has a co-substrate, that alterations in one of those factors . . .

Bhatnagar: Yes, the way this work was done, the only thing we took was a dialyzed extract; you don't really have cofactor problems here.

McLees: Well, that you know about.

Bhatnagar: Any cofactors that could be associated with the enzyme that we don't know about?

McLees: Because you're using very impure preps.

Bhatnagar: We are using a very impure prep, but the cofactors of the enzyme are small molecular weight compounds and they are dialyzed out.

McLees: I understand that; what I'm really getting at is that what you add back, you're assuming that you dialyze them away from the enzyme. What I'm just really saying is a more general fact: that we know of situations where enzymes are activated. It could well be that this enzyme is activated by one of these agents and you're just seeing an increased activation.

Bhatnagar: Well, that agent would have to come along with the enzyme from the lungs. If it is there, then it is still causing an activation of the enzyme and increased activity.

McLees: The reason I would like to make this point is that there are cases in lung associated with increased prolyl hydroxylase activity that as far as we can tell are not associated with increased turnover of collagens, and that's true in some pathologic states.

Bhatnagar: It would have to be a nondialyzable macromolecular factor that travels through all the steps.

McLees: I have no idea of mechanism, I just know that there are investigators who have reported increased prolyl hydroxylase activity in pathologic pulmonary states without increased turnover of collagens, or increased amounts of collagens.

Albert: What kind of state?

McLees: Some of the fibrotic lung disorders, and I'm sorry, I can't give you off the top of my head the specific pathologic state, but in lung fibrosis, my guess is that it's one of the idiopathic pulmonary fibroses.

Orthoefer: Aren't you talking about the same thing?

McLees: No, I don't think so. That's the other point here. We sort of assumed here that we are talking about pulmonary fibrosis as the pathology in this disorder. Is that true or is it not? If this is emphysema, then that is probably not a fair comparison at all.

Orthoefer: We're speculating on remodeling.

Comment: What is the pathology, do you see pulmonary fibrosis pathologically?

Orthoefer: No, it's not a major thing.

Dungworth: It's not a major feature of the lesion, no. It's an amount of increase in fibrous tissue around small airways that we haven't really got a good clue on yet because we haven't got any quantitative data. It's not the major feature of what we see.

McLees: Is the physiology compatible with fibrosis?

Comment: No.

McLees: Then I have to say I'm not sure that you are dealing with a fibrotic lung process.

Bhatnagar: I guess that's why there is no difference in collagen.

McLees: Well, in the human state, we are dealing with it and we still don't see that, but we have to take something as a base for lung fibrosis and I think it is important to establish. The base we use now, at least in humans, is physiology and pathology, and biochemically, as I say, we're not able to ...

Orthoefer: I don't think we can here either.

Bhatnagar: May I ask a very naive question? This is something that's completely foreign to me. I don't really understand lung structure and morphology. What exactly does constitute fibrosis? What does one see in a lung when one says it's a fibrotic lung? I see that term in a lot of places and I've tried to understand the pictures that come with that, but I don't understand the pictures.

McLees: I'm sure there are people like Dr. Kleinerman who could answer this better than I, but ...

Kleinerman: Maybe we ought to leave this for the time when we are discussing pathology, but I think, in general, one would see fibroblasts and the deposition of collagen in a number of areas. The deposition of collagen as defined morphologically by stains and morphologic characteristics.

Bhatnagar: Using morphologic techniques one does not really see basement collagen in the same way as one sees fibrous collagen. Early events in lung injury remove the basement membrane. What I would be able to measure biochemically as collagen, you will not be able to see. If this were replaced with a few fibers of type I collagen, for instance, you would see more collagen morphologically, even though there may be less collagen or no difference in the collagen content, as we saw here.

Kleinerman: We would be able to tell because basement membrane looks different than other types of collagen. It is possible that there could be a subtle change from one type to the other that wouldn't be distinguishable morphologically, but really, it's quite striking, the difference in basement membrane structure from the usual collagen.

Bhatnagar: This is what I wanted to know. When we see fibrosis we don't necessarily relate that to increased thickness of the basement membrane. Instead one sees the periodically banded collagen fibers.

Kleinerman: You can see it in that area and in other areas as well.

Bhatnagar: One other possibility that I did not mention here is that perhaps it is possible that in lesions, if one would call these things lesions, a part of the alveolar wall disappears, for instance. When we have increased collagen in one area and a total loss in another, then a technique like mine, where I can just take a fragment and hydrolyze it, may not be able to detect quantitative and qualitative differences.

Comment: That's right.

Bhatnagar: So there can be a number of different reasons why we didn't see these differences.

Orthoefer: Isn't it also true that basement membrane collagen is more compact, but you just looked at total collagen? Therefore, if you take a basement membrane which is more compact, a smaller unit of this would probably equal a large unit of collagen.

Bhatnagar: It certainly doesn't show up like nice fibrous collagen and other types which may form loose aggregates of fibers.

McLees: The problem here is that basement membrane collagen has never been biochemically found in lung. No one has isolated the basement membrane collagen from lung to my knowledge.

Bhatnagar: It seems to be there though.

McLees: We can't find it, and I mean we have looked.

Bhatnagar: What criteria have you used?

McLees: We've used specific isolation techniques for type IV collagen; we've used 3-hydroxyproline analysis and in the scheme that we've used, we've isolated types I, II, and III collagen from lung in an inclusive way where we keep material balance, and we're not able to distinguish it.

Bhatnagar: We didn't see any 3-hydroxyproline either and I wondered if the basement membrane collagen of the lungs is somewhat different.

McLees: Well, that may be, but if it is different it certainly does not meet the criteria where we normally classify it as a type IV collagen.

Bhatnagar: So little is known about what goes on in the lung anyway.

McLees: Well, that's true, but the point I wish to make is that in terms of basement membrane collagen as normally defined, (a highly glycosalated 3-hydroxyproline-containing molecule), there's no evidence supporting its presence in lung. Now, there may be a different type of basement membrane collagen that's not been isolated.

Kleinerman: Even when you look in the large airways, is that true?

McLees: We have looked everywhere. We have looked everywhere for that molecule and we believe there is a basement membrane collagen, we think that we may have it, I don't know.

Lee: This might muddy the water a little more, but Dr. Bhatnagar mentioned about the work of Hussain *et al.*[1] on the altered collagen metabolism following chronic exposure of rats to ozone, and recently Tierney's group down at UCLA ran similar experiments and obtained very similar data. Furthermore, these workers supplemented the experimental animals with vitamin E at 500, 50 and 10 ppm, and observed protective effects.

Bhatnagar: Were they looking at ozone or nitrogen oxide?

Lee: Ozone at 0.8 and 0.5 ppm for 4 days.

---

[1] *Hussain, M.Z.* et al. *1976. Ozone-induced pulmonary fibrosis: Synthesis of collagen in lungs in rats exposed to ozone. Life Sciences.*

5. THE EFFECTS OF AIR POLLUTANTS ON MEMBRANE LIPIDS

G. Rouser and R. Aloia

Abstract

The effects on membrane lipids of long-term exposure (68 months) of beagle dogs to raw and irradiated auto exhaust and various mixtures of oxides of nitrogen and sulfur followed by exposure to clean air (2½ to 3 years) were studied. No changes were found in brain or heart; an indication of small changes in liver phospholipids was apparent, and clear-cut changes were observed in the lung. Phosphatidyl ethanolamine from lung was reduced from 19.0% of the total phospholipid to 17.2% and some of the minor acidic phospholipids were elevated. No change was found in the levels of phosphatidyl choline or the other major phospholipid classes. Since the range of phosphatidyl ethanolamine in 15 lung samples was 17.7 to 20.3%, it is clear that the mean value for the experimental groups is outside of the normal range of variation. A mechanism for production of apparently permanent changes other than at the genetic level is not apparent.

Introduction

The effects of pollutants on membrane lipids that are essential for membrane function are important to define since membranes control the passage of substances into and out of cells, and function in impulse transmission. In principle, effects can be evaluated by analysis of whole organs or isolated membranes and organelles. In practice, analysis of whole organs is the only way that a large series of animals can be examined with reasonable effort, and it has the advantage that membrane lipids are not altered by isolation procedures. In this report, the results of a study of the effects of pollutants on the phospholipids of brain, heart, liver and lung are presented.

Materials and Methods

Beagle dogs were maintained and exposed to polluted air as described elsewhere (1). Organs from the animals were removed immediately after sacrifice and stored at −20°C. Lipids were extracted with chloroform/methanol, and nonlipid contaminants were removed by Sephadex column chromatography. Phospholipids were separated by two-dimensional thin-layer chromatography, and their molar amounts determined by phosphorous analysis as previously described (2,3,4).

Results and Discussion

The data for heart lipids (Table 1) show that the controls and the experimental groups are closely similar. The small range of variation of the values shows the high degree of reproducibility of the analytical methods that is also shown by the standard deviations (in the 1 to 2% range for major components). Phospholipids of brain (data not shown) do not show changes in the exposed animals and the range of variability is slightly less than that for heart lipids.

Table 1. Heart Lipids (Mean Values)[a]

Lipid[b]	Control range	Experimental groups							All experimental[c]	All control[d]
		R	I	SO_x	$R + SO_x$	$I + SO_x$	$NO_L + NO_{2H}$	$NO_H + NO_{2L}$		
TL	5.12–10.49	6.79	6.29	5.93	6.35	6.91	4.86	4.76	5.98 ± 0.86	7.52 ± 1.73[e]
TPL	1.78– 4.44	2.03	2.12	2.06	2.14	1.66	2.19	2.12	2.04 ± 0.18	2.25 ± 0.78
PC	38.9 –42.7	42.1	40.1	41.6	41.5	40.1	40.3	40.7	41.0 ± 0.8	41.1 ± 1.4
LPC	1.0 – 1.8	1.6	2.1	1.6	1.4	1.7	1.8	1.7	1.7 ± 0.2	1.4 ± 0.3
PE	28.2 –29.7	28.6	29.3	29.0	28.8	28.9	29.6	29.0	29.0 ± 0.3	29.1 ± 0.6
LPE	0.8 – 2.0	2.5	1.5	1.4	1.6	1.4	1.3	1.2	1.6 ± 0.4	1.2 ± 0.3
SPH	3.7 – 5.2	4.6	4.1	4.1	3.9	4.4	4.6	4.3	4.3 ± 0.3	4.3 ± 0.5
PS	2.3 – 3.6	2.7	2.4	3.6	2.7	2.5	2.3	2.5	2.7 ± 0.4	2.8 ± 0.3
PI	3.6 – 4.3	4.0	4.2	3.8	3.8	4.3	3.9	4.2	4.0 ± 0.2	4.0 ± 0.2
DPG	7.6 –10.1	8.2	6.8	10.7	9.7	9.5	10.4	10.3	9.4 ± 1.4	8.9 ± 0.8
LDPG	1.5 – 2.4	2.1	2.3	1.7	2.1	1.8	1.8	1.9	2.0 ± 0.2	1.8 ± 0.3
PA	0.3 – 0.8	0.4	0.3	0.4	0.3	0.9	0.4	0.3	0.4 ± 0.2	0.6 ± 0.2
Misc	3.7 – 6.2	3.3	6.9	3.1	4.1	4.6	3.5	3.9	4.2 ± 1.3	5.0 ± 0.8

[a] Quantities expressed in all tables:

TL = total lipid = % fresh weight

TPL = total polar lipid = m moles/100 g fresh weight

Individual lipid classes = mole % values based on recoveries between 97 and 101%

[b] Abbreviations used:

TL = total lipid; TPL = total polar lipid; PC = phosphatidyl choline; LPC = lysophosphatidyl choline; PE = phosphatidyl ethanolamine; LPE = lysophosphatidyl ethanolamine; SPH = sphingomyelin; PS = phosphatidyl serine; PI = phosphatidyl inositol; DPG = diphosphatidyl glycerol; LDPG = lysodiphosphatidyl glycerol; PA = phosphatidic acid; LBPA = lysobisphosphatidic acid; Misc = miscellaneous

[c] Mean and standard deviation of all experimental groups.

[d] Mean and standard deviation of all control groups (n = 10).

[e] Significant difference at 2.5% level between mean values of control and experimental groups (TL; Student's t-test).

Liver lipids (Table 2) present a less clear-cut situation. The mean values for experimental groups 1 and 2 (R and I) in particular suggest a small effect on phosphatidyl ethanolamine. Each sample in these groups was analyzed separately. Although the results do not show any obvious differences, some of the phosphatidyl ethanolamine values are below the lowest value found for controls. Also, a number of minor components could be seen more prominently on thin-layer chromatograms of the experimental groups. Additionally, the total lipid fraction (phospholipids and neutrals) was increased significantly in the experimental groups (Table 2).

In contrast to the other organs, the phospholipids of lung showed definite differences between controls and all experimental groups. The percentage of phosphatidyl ethanolamine is 90% of the control mean value. The values for minor acidic phospholipids in general, and in particular the levels of lysobisphosphatidic acid and phosphatidyl glycerol (significant at the 1% level; Table 3), are increased. The changes are relatively specific since values for the other major lipid classes were not changed. It should be noted that the mean value for phosphatidyl ethanolamine of the experimental groups (17.2%) is outside the normal range of variation.

A mechanism for the production of permanent changes other than alteration at the genetic level is not apparent. It seems probable that the production of some particular molecular species of phosphatidyl ethanolamine in lung is impaired. In this regard it is interesting to note that exposure to ozone was found to decrease linoleic acid and increase arachidonic acid in lung lipids (5). The changes are similar to those produced by vitamin E deficiency (6,7). A logical next step is the determination of the fatty acid composition of each of the phospholipids of lung. It is to be noted that analysis of pure membrane preparations and organelles might disclose changes that are not apparent from whole organ data.

References

1. Stara, J., K. Busch, R.H. Hinners, and J.K. Burkart. 1979. Study overview, rationale, experimental design, experimental facilities, and exposure atmospheres. Chapter 1 of this book.
2. Rouser, G., G. Kritchevsky, and A. Yamamoto. 1967. Column chromatographic and associated procedures for separation and determination of phosphorized and glycolipids. Pages 99-162 *in* G.V. Marinetti and Marcel Dekker, eds., Lipid chromatographic analysis, vol. 1.
3. Rouser, G., S. Fleischer, and A. Yamamoto. 1970. Two-dimensional thin-layer chromatograph separation of polar lipids and determination of phospholipids by phosphorous analysis of spots. Lipids 5: 442-444.
4. Rouser, G. 1973. Quantitative liquid column and thin-layer chromatography of lipids and other water-soluble substances, elution selectivity principles, and a graphic method for pattern analysis of chromatography data. J. Chromatographic Sci. 2: 60-76.
5. Menzel, D.B., J.N. Roehm, and S.D. Lee. 1972. Vitamin E: The biological and environmental antioxidant. J. Agr. Food Chem. 20(3): 481-486.
6. Witting, L. 1967. Effects of antioxidants deficiency on tissue lipid composition in rats. IV. Peroxidation and interconversion of polyunsaturated acids in muscle phospholipids. Lipids 2: 109-113.
7. Bernhard, K., S. Leisinger, and W. Pedersen. 1963. Vitamin E und Arachidonsaure-Bildung in Der Leber. Helv. Chim. Acta 46: 1767-1772.

Table 2. Liver Lipids (Mean Values)[a]

Lipid[b]	Control range	Experimental groups								
		R	I	SO_x	R + SO_x	I + SO_x	$NO_L + NO_{2H}$	$NO_H + NO_{2L}$	All experimental[c]	All control[d]
TL	4.04– 7.22	6.02	5.80	5.54	6.86	6.70	6.94	6.34	6.31 ± 0.55	5.63 ± 0.79[e]
TPL	2.74– 4.04	3.56	3.86	3.59	4.02	3.68	3.56	3.82	3.73 ± 0.18	3.56 ± 0.41
PC	45.9 –50.4	50.2	49.3	49.5	48.2	50.9	49.3	48.6	49.4 ± 0.9	48.4 ± 1.5
LPC	1.5 – 2.5	1.9	1.7	1.8	1.8	1.6	1.8	2.0	1.8 ± 0.1	2.0 ± 0.3
PE	24.9 –27.4	24.7	27.2	25.6	25.2	24.4	25.3	25.5	25.4 ± 0.9	26.0 ± 1.0
LPE	0.6 – 1.0	0.5	0.2	0.4	0.8	0.4	0.0	0.6	0.5 ± 0.2	0.7 ± 0.1
SPH	4.1 – 5.7	4.6	4.6	4.7	4.8	4.5	5.1	4.4	4.7 ± 0.2	4.6 ± 0.4
PS	2.3 – 4.1	3.0	2.5	3.8	3.8	3.7	3.7	3.8	3.5 ± 0.5	3.6 ± 0.5
PI	6.2 – 8.3	7.7	7.6	7.1	7.8	7.8	7.8	7.4	7.6 ± 0.3	7.4 ± 0.5
DPG	3.4 – 4.6	3.9	4.2	3.9	4.0	3.2	3.8	3.6	3.8 ± 0.3	3.8 ± 0.4
LDPG	0.1 – 0.6	0.5	0.1	0.6	0.7	0.4	0.6	0.7	0.5 ± 0.2	0.4 ± 0.1
PA	0.6 – 1.1	0.7	0.4	0.7	0.9	0.7	0.7	0.8	0.7 ± 0.2	0.8 ± 0.2
Misc	1.9 – 3.0	2.2	2.2	2.0	2.0	2.4	2.0	2.0	2.1 ± 0.2	2.3 ± 0.4

[a] Quantities as for Table 1.

[b] Abbreviations as for Table 1.

[c] Mean and standard deviation of experimental groups.

[d] Mean and standard deviation of control groups.

[e] Mean values of control and experimental groups are significantly different at the 5% level (TL; Student's t–test).

Table 3. Lung Lipids (Mean Values)[a]

Lipid[b]	Control range	Experimental groups								
		R	I	SO_x	R + SO_x	I + SO_x	$NO_L + NO_{2H}$	$NO_H + NO_{2L}$	All experimental[c]	All control[d]
TL	1.57- 4.01	2.74	2.78	2.63	2.73	2.84	2.99	2.97	2.81 ± 0.13	2.78 ± 0.50
TPL	1.12- 2.60	2.08	2.10	2.07	2.11	2.17	2.29	2.29	2.15 ± 0.11	2.05 ± 0.41
PC	33.6 -47.1	44.5	41.4	37.9	44.1	39.9	43.0	43.0	42.3 ± 2.3	42.0 ± 3.1
LPC	1.0 - 2.1	1.2	1.4	1.3	1.4	1.3	1.4	1.3	1.3 ± 0.1	1.3 ± 0.2
PE	17.7 -20.3	16.9	17.5	18.0	17.1	17.8	16.6	16.7	17.2 ± 0.5	19.0 ± 0.8[e]
SPH	13.9 -18.3	14.8	16.1	16.9	15.2	17.1	15.7	15.7	15.8 ± 0.8	15.9 ± 1.3
PS	8.5 -10.6	8.2	9.1	9.7	8.1	9.3	8.8	8.7	8.8 ± 0.6	9.1 ± 0.5
PI	1.8 - 3.3	3.3	2.7	2.6	3.4	2.7	3.1	3.3	3.1 ± 0.3	2.8 ± 0.4
DPG	0.8 - 1.3	0.9	1.0	1.1	1.1	1.0	1.1	1.0	1.0 ± 0.1	1.0 ± 0.1
PA	0.7 - 2.3	0.7	1.5	1.2	0.7	1.8	0.9	1.2	1.1 ± 0.4	1.4 ± 0.5
LBPA	1.4 - 5.2	3.8	3.7	3.6	3.4	3.3	3.5	3.6	3.6 ± 0.2	2.9 ± 1.2
PG	2.1 - 3.9	3.1	3.7	4.7	3.5	4.0	3.8	3.5	3.8 ± 0.5	3.0 ± 0.5[f]
Misc	1.4 - 3.9	2.6	2.1	3.1	2.1	1.8	2.2	2.1	2.3 ± 0.4	2.0 ± 0.6

[a] Quantities as for Table 1.

[b] Abbreviations as for Table 1.

[c] Mean and standard deviation of experimental groups.

[d] Mean and standard deviation of control groups.

[e] Significant difference at the 0.1% level between mean values of control and experimental groups (PE; Student's t-test).

[f] Significant difference at the 1% level between mean values of control and experimental groups (PG; Student's t-test).

Asilomar Conference Discussion

Lee: As you noted already, in the initial phase of this study, not much biochemical work was included, for two good reasons. First of all, even though our facilities might be the best in the world, they still are limited, and we could not include sufficient numbers of animals to allow serial sacrifice to do biochemical work. Secondly, perhaps more importantly, a lot of information that's available now, at low level of exposure to various pollutants, mainly ozone and NO_2, down to 0.1 ppm levels, was not available back in 1964 and 1965. I think, even though some of the techniques might have been available, there was not enough communication between basic researchers and applied researchers and the techniques were simply not being used. It would have been ideal if the funds and facilities were available to accommodate serial sacrifices to examine various changes throughout the duration of exposure. In the recent days the biochemists are talking to morphologists, and morphologists are talking to biochemists. I literally mean that, and a lot of the credit goes to the Davis School, SRI, and UCLA groups. They found biochemical changes at very low concentrations, mainly I guess the SRI group saw morphological changes at very low concentrations, they now can relate the two different changes. In the past, when the biochemists gave you some kind of dose-response, people in Washington, or even in Cincinnati, came back and said, "What does that mean?". Still we were told to do the dose-response studies. I think this kind of difficulty is gradually disappearing. At least, I hope so. Getting back to Dr. Bhatnagar's findings in terms of collagen synthesis and Mustafa's finding and Hussain's work with ozone exposure, there is initial depression. I'm talking about some of the subcellular marker enzymes that we investigated. They showed initial depression and then increased coincidental with cellular proliferation at about day 3 or 4. I think it's very important to include this kind of parameter for any kind of future studies.

Since the time is getting quite late, I'm not going to go into any kind of specific recommendation. Hopefully, I will have a chance to insert my recommendation during the last day's session, but I hope that all the presentations during the next two days will point to additional studies of low-level, long-term effects. I wish that a group of experts like the present company would plan that study. Hopefully, we could get stable funding for the entire period, so that the researchers wouldn't have to worry about funding every year, thus enabling them to concentrate on high quality research. I think I'll stop right there.

Stara: I wonder if you would mention the small experiments you performed at the beginning of the study; I mean the sulfur content of the blood and the vitamin C.

Lee: During the early part of exposure of the dogs, namely, three sampling periods, December 1965 and April 1966 and September 1967, we looked at total sulfur content of the blood and vitamin C, in the hope that we could use them as indices of change in sulfur metabolism or oxidative metabolism, but we did not see any difference among the groups and we discontinued the effort. One interesting aspect, a very small sidelight, was that we shipped six blood samples from these dogs, three controls and three NO_2-exposed blood samples, to John Rowlands at Southwest Research Institute. We sent the samples completely blind and he segregated them into two different groups based on his EPR analyses. The control group did not give any kind of triplet signal and the NO_2-exposed groups gave clear triplet signals. With only that much information we did not get funding to continue that type of work and had to stop.

Kleinerman: Would you explain the significance of these preliminary findings? What do you think they mean?

Lee: You mean this EPR signal? I think it means that NO_2 exposure does cause the free radical formation and EPR techniques were able to pick it up.

Rouser: Dr. Lee showed me some of the lipid data that he selected. What was that on, rats?

Lee: Yes, the kind of PE change Dr. Rouser mentioned earlier in conjunction with that particular experiment. Actually, about the same time we exposed some rats to NO_2 at 1 ppm for 2 weeks and then we washed the lungs out and looked at the lipid profile of the endotracheal lavage, as a quick means of looking at the change in surfactant. There was a definite drop in PE and increase in phosphotidyl choline. The latter change was not seen by Dr. Rouser. The marked drop in PE in both experiments agreed.

Rouser: Yours was actually larger by quite a bit than we....

Lee: Yes.

McLees: Another interesting ramification of that is that the spin label which was used is the nitroxide radical which is NO. It may be that the EPR signal is coming from degradation of the NO_2 that's attached to the lipid. Maybe it is generating an *in vivo* spin label with that.

Orthoefer: Would you expect a membrane lipid change in anything other than the lung in normal animals that do not have some overt disease?

Rouser: No, as a matter of fact, Dr. Lee had to spend, as I recall, roughly 3 years talking me into doing the study at all because I felt that there was only one way that we could see a permanent change and that would be to affect the genetic apparatus. I felt that this didn't seem too likely in organs generally, and there was a slight possibility that the low levels used over long periods might do the job because of the overall importance even of a negative result, and particularly since Dr. Lee was so persistent, I finally agreed to do the research. Furthermore, I was so certain there would be no effect that I didn't bother even to look at the data sheets to examine them for possible significance except to make sure that my technicians had done all their jobs correctly. I was quite surprised that there was anything. I didn't anticipate finding anything at all. I had no basis, no reason to think so, and I'm still a bit surprised. My big question at this point, though, is how long really does it take to make such a change? I suspect that in the growing animal, and this is why I would emphasize the developmental aspect, these changes would occur over a shorter time period. I neglected to mention a very important point — that these are time-coordinated. It appears from studies of severe protein deficiency that in brain you can induce a deficiency and then can put an animal back on a sufficient diet and it seems to regain everything. However, it does not regain the morphologic and perhaps not the proper functional features, so that developmental changes may be significantly affected. I think that's one of the reasons I'd like to see them studied. Now I'm curious, how long does it take to produce a change that's going to be permanent? In other words, expose the animals for short periods of time and then let them go for a while and see what happens. I really think now that perhaps it doesn't take that long. You have to realize, too, that all of the organs undergo development changes. Not just in terms of weight increases but the composition of all the major components is grossly different. DNA and RNA drop down to about 20% of the starting value in the fetal tissue, the protein changes, the lipid goes up always in each organ — the total amount of polar lipid — and these seldom level off absolutely flat. In the human brain, this thing just continues at a progressively decreasing rate throughout life, you see, and so there are really very large changes. Our problem has been how to analyze the data. When you plot them incorrectly, there is a huge data spread, which you don't see when you use the correct function. I think we can analyze the data much better now.

Lee: Another thing that we should have looked at was the age-related fluorescent pigments on which Dr. Tappel's group at the University of California at Davis had been working. Perhaps this was due to Tappel's preference to grants over contracts, but the fluorescent pigments, at least, in three different organs (heart, brain and testes), should have been investigated.

Stara: I just wonder if I could ask the three of you, Dr. Orthoefer, Dr. Lee, and Dr. Bhatnagar, what tissues do we have left? We have still some of the tissues, right? You have it frozen, or what is the situation with the tissues that were submitted for biochemical examination to your laboratory?

Bhatnagar: We have a very small amount of tissue, if any at all, and not from all the animals.

Rouser: We actually have a goodly supply of tissues. We took organs which had not been analyzed. I had made this mistake before of not taking everything. We have aorta and so on, and we have quantities of everything that we had run so that there is anything that's preserved. We had to put these in a -20°C box. Now that means that many enzymes are not going to be present, unfortunately.

Stephens: The changes that you observed in the lipids in the lungs were in the phospholipid fraction, were they not? The lamellar bodies in the type 2 cells have, I believe a considerable amount of phospholipids in them. Does someone who has worked with the lamellar bodies have specifics?

Rouser: I know what's been reported about this lipid composition.

Stephens: They do have phospholipid components.

Rouser: Oh, yes. Well, there are two reports; one of them is correct, I think. It says that there is a lipid controlling ratio 4:1 similar to that in mice. The other one says 12:1 but I'm quite certain that there's just no way that that could be correct. I think that 4:1 though is quite probably correct. Of course, the lipid class composition has not to my knowledge been reported.

Stephens: I just wondered if you would speculate on what, or if you have speculated on what the factor is that causes the change in the lung. Could it be a change in the synthesis of the phospholipid component of the lamellar body?

Rouser: Yes, it could be. The only mechanism I can visualize ... I've racked my brain, I've talked to every geneticist, read every metabolic regulation guide around our place, and the only mechanism I can visualize is one affecting the genetic apparatus; that is, to make a permanent change.

Albert: Couldn't you just get a difference in the balance of the cell types?

Rouser: Briefly, in quick passing, I realize I referred to that, and said that I feel that this is most likely excluded by the types of numbers we got because in every case of that type that I know of, there is change in all of the phospholipid classes. In other words, when certain membranes do in fact have more sphingomyelin and some less phosphotidyl choline (PC), the fact that the PC and sphingomyelin values in those animals were all exactly the same rules out, for practical purposes, a variation in the relative proportion of membranes of a single cell. You have, however, hit upon one of the very obvious possibilities as one of the things that can happen to you in whole organ analysis. I might add there is one more thing where there could be changes that are significant. For one particular membrane that we would miss . . . it may very well be the case, for example in lipids, that maybe what we're seeing in liver is a change that would be prominent in say, endoplasmic reticulum or in the mitochondria, and can't prove to be significant in whole organ analysis.

Stephens: The changes you saw were equal in all experimental animals?

Rouser: In the lung they were essentially the same, yes.

Stephens: It didn't matter what the exposure was, the change was essentially the same.

Rouser: They may have differed slightly. But by my estimate, I don't think it would be worth the time and effort to analyze the individual samples to show the exact nature of that range.

Stephens: Many of us have speculated, of course, about the nitrous acid formed from NO_2 in exposed animals and its potential to cause genetic change, but if all of the differences in the experimental exposures produce essentially the same kind of change in the lipid component of the membranes, then the change must be relatively nonspecific.

Rouser: Yes, from the standpoint of the cause, but from the standpoint of effect, I would suspect that if a particular set of fatty acid combinations that for some reason simply are not made in the proper manner (and of course this is something we could show, by determining the fatty acid composition and the molecular species distribution of these lipids) — it's almost certain that it would have to be a single type of membrane lipid. The levels are just so, cut down to the same amount and I suspect that it's a particular type of phospholipid that somehow gets wiped out. However, is it significant physiologically? The only kind of answer we could give to that, of course, is that we don't know. But we would expect on the basis of what we know about the specificity of lipid protein-interaction that it very likely could be significant to cause definite changes in structure/function relationships.

McLees: Our time is long past up. Thank you very much.

6. PULMONARY AND CARDIOVASCULAR PHYSIOLOGY STUDIES DURING EXPOSURE

T.R. Lewis and W.J. Moorman

A limited amount of data exists for cardiopulmonary responses to well-defined exposure to automobile exhaust or simple mixtures of other predominant air pollutants. Furthermore, most investigations into the effects of air pollution have emphasized major anatomic, subsystem failure, as denoted primarily by pathological lesions, instead of altered ranges of performance. The studies which have dealt with urban air pollution in Los Angeles or extreme air pollution episodes are of limited value in accurately assessing responses because of the absence of essential analytical data regarding exposure. These exposures are also difficult to interpret as a result of the inability to deal with the human factors of age, sex, smoking, occupational exposures, genetics, nutrition, etc. Animal experimentation is a necessary part of health effects data because of the controlled, analytical approach.

Swann and Balchum (1) reported significant increases in total expiratory flow resistances when guinea pigs were exposed to oxidant levels of either 0.59 mg/m^3 (approximately 0.3 ppm) or when approximate atmospheric concentrations of 45.8 mg/m^3 (40 ppm) carbon monoxide, 2.25 mg/m^3 (1.2 ppm) of oxides of nitrogen and 10.6 mg/m^3 (16 ppm) of hydrocarbons were present. Severe air pollution episodes were reported by Wayne and Chambers (2) to produce transient increases in pulmonary resistance in aged guinea pigs, but no demonstrable chronic or cumulative effects. These responses were most evident on two successive days when the oxidant level exceeded 0.98 mg/m^3 (0.5 ppm). Emik and co-workers (3) found that guinea pigs reared in filtered air were significantly more responsive to a challenge dose of 0.98 mg/m^3 ozone than their experimental counterparts reared in ambient air.

Hueter *et al.* (4) measured total airway resistance in guinea pigs every 16 weeks for 20 months. These guinea pigs were under a 7-day-per-week, 16-hour-per-day exposure regimen to either diluted natural or simulated photochemically reacted auto exhaust; however, the total airway resistance measurements were performed while the animals were breathing room air. No alterations in airway resistance were demonstrable. Murphy (5), however, reported elevations in airway resistance while guinea pigs were breathing low concentrations of auto exhaust. The elevated airway resistance disappeared when the animals were breathing room air. Murphy (5) also reported elevated carboxyhemoglobin values in animals exposed to irradiated auto exhaust versus auto exhaust and also in rats and mice breathing a mixture of carbon monoxide and ozone versus carbon monoxide alone. This observation of an apparent increase in carboxyhemoglobin when oxidants are present warrants consideration.

Although heart disease is the leading cause of mortality in the United States (6) and air pollutants are at historically high levels (7), no well-controlled experimental studies have been performed to assess the chronic cardiovascular effects of long-term exposure to ambient levels of air pollutants. Individual components of ambient air pollution have been demonstrated to have cardiovascular effects: carbon monoxide inhalation has been associated with myocardial ischemia and infarction (8, 9); ozone inhalation with discrete myocardial nuclear lesions in mice (10); and sulfur oxides with human myocardial infarction (11). These physiological investigations were initiated due to lack of objective studies to assess cardiopulmonary performance in animals subjected to long-term, low-level exposure to simple and complex mixtures of air pollutants.

Methods

General experimental design features such as chamber design and function, number of experimental subjects, test atmospheres, etc., have been described in previous papers. The atmospheres are presented in Table 4 of Chapter 2.

Pulmonary function testing was performed prior to initiation of the exposure regimen and at 18, 36 and 61 months of exposure. Cardiovascular status studies were conducted following 48 and 54 months of exposure to the test atmospheres. Pre-exposure pulmonary function values were determined on anaesthetized dogs utilizing a capacitance respirometer. Variables measured were respiration rate, tidal volume and inspiratory and expiratory flow rates.

Dogs were tested at 18 months of exposure for single-breath diffusion capacity, pulmonary compliance and total expiratory resistance (D_L, C_L, and R_L) employing the methodologies and conditions reported by Vaughn *et al.* (12). These same tests were performed with the addition of single-breath nitrogen washout and all lung volumes at both 36 and 61 months of exposure. At 61 months tests for maximum breathing capacity (MBC), peak expiratory flow rate (PEF), pulmonary membrane diffusion (D_M), and pulmonary capillary blood volume (Q_C) were added. The 18- and 36-month studies were performed on anaesthetized dogs using positive pressure introduction of test gases via a syringe. At 61 months all directly performed volumes and capacities as well as MBC and PEF were obtained by use of a plethysmograph-respirator. In this system the introduction of test gases and inspiratory and expiratory maneuvers are done passively, i.e., air enters and leaves the lungs by mechanical action on the chest and not on the airway. Details of the experimental methodologies and conditions for pulmonary function testing at 36 and 61 months can be found in the article by Lewis *et al.* (13).

In order to assess the cardiovascular status of each beagle dog, three electrocardiograms were performed on different days within a 3-week period. Because of the variability of ECG's on any one beagle, resting ECG changes were not considered significant unless they occurred repeatedly in all three ECG's in a given sampling period. Furthermore, only those ECG abnormalities which persisted from the time of appearance were considered to be significant.

An attempt was made to obtain an objective interpretation of the ECG data. A randomly selected group of all resting ECG's from 31 dogs was interpreted independently by a canine electrocardiographic consultant, D.K. Detweiler, University of Pennsylvania.

Other tests to determine cardiovascular status including vectocardiograms, post-exercise (swimming) ECG's, selected blood pressures and phonocardiograms were also performed. Details of experimental methodologies and conditions can be found in the publication by Bloch *et al.* (14). Two other tests on cardiovascular status of beagles were conducted. Details of the methodologies for determining the relative incidence of widened QRS complexes and blood rheology can be found in other papers prepared by Bloch *et al.* (15, 16).

Results

At 18 and 36 months of exposure, no statistically significant treatment effects were detectable by the analysis of variance for any pulmonary function variable. The pulmonary function means by treatment and variable for 18 and 36 months are given in Tables 1 and 2. At 61

Table 1. Means and Standard Deviations of Body Weight and Pulmonary Function Tests — 18 months

Atmosphere	No. animals	Weight (kg)	DL (ml/mm Hg/min)	CL (ml/cm H_2O)	RL (cm H_2O/l/sec)
CA	19	9.6 ± 2.0	6.95 ± 1.36	37.3 ± 11.6	2.15 ± 0.95
R	12	11.5 ± 2.7	7.19 ± 2.09	37.0 ± 12.8	2.32 ± 1.56
I	12	10.3 ± 3.2	6.58 ± 1.48	33.5 ± 10.0	3.06 ± 1.99
SO_x	12	10.7 ± 2.9	7.33 ± 1.74	35.7 ± 11.9	2.73 ± 1.54
R + SO_x	11	10.0 ± 2.9	8.49 ± 2.30	41.7 ± 13.4	1.99 ± 1.42
I + SO_x	12	11.5 ± 2.3	6.89 ± 1.20	36.9 ± 11.5	2.74 ± 1.30
$NO_L + NO_{2H}$	12	10.2 ± 1.8	6.71 ± 0.85	37.3 ± 7.6	2.49 ± 1.27
$NO_H + NO_{2L}$	12	11.0 ± 2.4	7.53 ± 1.01	34.6 ± 5.3	2.71 ± 1.20

Key: DL = Diffusing capacity for lung
CL = Compliance of lung
RL = Resistance of lung

Table 2. Pulmonary Function Means by Treatment and by Variable at 36 Months of Exposure

	CA	R	I	SO_x	R + SO_x	I + SO_x	NO_L + NO_{2H}	NO_H + NO_{2L}	SD(E)[a]
CL dyn (ml/cm H_2O)	46.6	32.1	41.9	43.3	39.6	40.6	49.6	41.6	12.2
CL st (ml/cm H_2O)	46.0	44.2	48.9	46.9	45.9	43.9	52.9	45.9	11.4
$\frac{CL}{FRC}\left(\frac{ml/cm\ H_2O}{ml}\right)$	0.15	0.13	0.14	0.12	0.12	0.13	0.14	0.13	0.03
$RL_{200}\left(\frac{cm\ H_2O/liter}{sec}\right)$	1.28	1.67	1.20	1.15	1.45	1.33	1.75	1.32	0.09
$DL_{CO}\left(\frac{ml\ CO/min}{mm\ Hg}\right)$	7.28	7.44	7.28	7.59	7.47	7.38	7.06	7.39	1.27
$\frac{DL_{CO}}{TLC}\left(\frac{ml\ CO/min/mm\ Hg}{ml}\right)$	0.008	0.007	0.008	0.007	0.008	0.008	0.007	0.007	—[b]
RV/TLC (%)	20.8	19.3	19.6	20.5	20.2	20.1	19.5	19.4	3.5
FRC (ml)	314	332	305	344	319	302	358	310	61
ERV (ml)	132	137	124	143	129	119	156	118	47
RV (ml)	190	195	181	209	197	186	202	192	33
VC (ml)	770	837	755	781	805	802	864	811	114
TLC (ml)	952	1029	939	1035	992	982	1067	1003	154
IC (ml)	636	697	634	691	663	679	708	693	117
N	19	11	11	11	10	12	12	11	

[a] Standard deviation for replicate animals (experimental error).

[b] The value was too small to be presented.

Key: *CL dyn* = *Dynamic compliance of lung*
CL st = *Static compliance of lung*
$\frac{CL}{FRC}$ = *Specific compliance divided by functional residual capacity*
RL_{200} = *Resistance of lung measured at 200 ml/sec flow*
DL_{CO} = *Diffusing capacity of lung for carbon monoxide*
$\frac{DL_{CO}}{TLC}$ = *Diffusing capacity divided by total lung capacity*
RV/TLC = *Residual volume divided by total lung capacity (hyperinflation index) expressed as percent*
FRC = *Functional residual capacity*
ERV = *Expiratory reserve volume*
RV = *Residual volume*
VC = *Vital capacity*
TLC = *Total lung capacity*
IC = *Inspiratory capacity*
N = *Number of animals per treatment*

months of exposure the ANOVA did reveal significant differences in functional status among treatments. The pulmonary function means by treatment and variable are given in Table 3.

Those dogs receiving auto exhaust (R and I) had higher diffusing capacities than those receiving mixtures of oxides of nitrogen (9.91 vs. 8.13 ml CO/min/mm Hg/ml). A significant increase in the pulmonary capillary blood volume was demonstrated in dogs receiving auto exhaust when compared to those not receiving auto exhaust (19.8 vs. 17.2 ml, respectively). The difference here is the result of the higher concentration of CO in the auto exhaust treatments. Dogs receiving auto exhaust, auto exhaust plus oxides of sulfur, and NO_H + NO_{2L} demonstrated moderately elevated residual volumes (267, 280, 258 ml vs. 232 for CA). The irradiated auto exhaust treatment produced less uniform distribution of inspired air, whereas the oxides of sulfur appeared to improve distribution (1.8% N_2 vs. 1.4% N_2, and 1.1% N_2 vs. 1.4% N_2, respectively).

A comparison of the incidence of individual dogs functioning at levels at the lower spectrum of normality based on clinical criteria revealed that dogs receiving NO_L + NO_{2H} had lower diffusing capacity values than the controls after 36 months of exposure. The incidence of lower diffusing capacities for the exposed NO_L + NO_{2H} versus control dogs was 5 of 12 versus 0 of 18, respectively. This was the first treatment-related effect to be statistically identified in this study.

The following results of 61 months of exposure were found to be statistically significant when the clinical entries and subsequent chi-square analyses were employed. Dogs that received irradiated auto exhaust (I and I + SO_x) had higher total expiratory resistances than their comparable controls (CA and SO_x), 9 of 21 vs. 0 of 28, respectively. These data were statistically significant at the 0.0001 probability level. A decrease in peak expiratory flow rate was noted in dogs receiving the NO_L + NO_{2H} treatment when compared to CA subjects (5 of 11 vs. 1 of 18, respectively), $P = 0.018$. Analysis of the $D_{L_{CO}}$ values did not detect treatment differences and was again probably due to the failure to compensate for the biological variation inherent in the experimental population. The $D_{L_{CO}}$ was adjusted to a percent of the predicted value which was markedly different from dogs receiving NO_L + NO_{2H}. The respective frequencies were 0 to 18 for CA dogs and 5 of 11 for NO_L + NO_{2H} dogs, $P = 0.0004$. The prediction formula employed for $D_{L_{CO}}$ was the one proposed by Giammona and Daly (13) in which $D_{L_{CO}} = 0.0058\ TLC + 1.7$. Such an adjustment is similar to $D_{L_{CO}}/TLC$ ratio and the results are essentially identical. Dogs receiving $R + SO_x$ had a higher mean RV/TLC ratio at 61 months of exposure than dogs in CA (6 of 10 vs. 1 of 18, respectively), $P = 0.003$. This result is smaller than the mean in the three treatments $R + SO_x$, R, and NO_H + NO_{2L} obtained by analysis of variance.

The incidence of abnormal ECG's, static and post-exercise, are reported in Table 4. Consistently positive static ECG diagnoses were made of one dog exposed to clean air and irradiated auto exhaust during both sampling periods and in one dog exposed to oxides of sulfur alone and in combination with auto exhaust in the second sampling period. Since the left axis deviation could be explained on the basis of mitral insufficiency, the abnormality of the ECG of the dog exposed to clean air was discounted from consideration. Hence, there was an incidence of 4.1% (3 of 73) of consistent ECG abnormalities in dogs exposed to air pollutants versus 0 of 18 in controls. One dog exposed to irradiated auto exhaust and auto exhaust had bradycardia, a diagnosis with nonspecific implications. The findings of LAD and elevated

Table 3. Pulmonary Function Means by Treatment and by Variable at 61 Months of Exposure

	CA	R	I	SO_x	$R + SO_x$	$I + SO_x$	$NO_L + NO_{2H}$	$NO_H + NO_{2L}$	SD(E)[a]
CL dyn (ml/cm H_2O)	35.7	36.3	35.8	34.3	40.2	32.9	41.0	38.6	12.0
CL st (ml/cm H_2O)	38.8	43.9	45.0	41.5	48.9	47.7	48.3	41.9	15.9
$\frac{CL}{FRC} \left(\frac{ml/cm\ H_2O}{ml}\right)$	0.10	0.09	0.10	0.10	0.10	0.09	0.10	0.10	0.03
$RL_{200} \left(\frac{cm\ H_2O/liter}{sec}\right)$	2.94	2.87	3.14	2.85	2.48	2.53	2.72	2.73	0.85
$DL_{CO} \left(\frac{ml\ CO/min}{mm\ Hg}\right)$	8.63	10.40	9.36	9.31	9.71	8.74	7.67	8.89	2.63
$\frac{DL_{CO}}{TLC} \left(\frac{ml\ CO/min/mm\ Hg}{ml}\right)$	0.008	0.009	0.009	0.008	0.009	0.007	0.007	0.008	0.002
$D_m \left(\frac{ml}{min/mm\ Hg}\right)$	25.3	16.6	20.8	23.1	24.4	24.1	22.1	20.4	7.1
Q_c (ml)	16.6	19.8	18.6	18.0	20.2	20.5	18.5	16.5	5.5
RV/TLC (%)	21.0	22.3	20.3	18.0	24.7	21.8	20.0	22.0	4.0

[a] Standard deviation for replicate animals (experimental error).

Key: *CL dyn* = *Dynamic compliance of lung*
CL st = *Static compliance of lung*
$\frac{CL}{FRC}$ = *Specific compliance divided by functional residual capacity*
RL_{200} = *Resistance of lung measured at 200 ml/sec flow*
DL_{CO} = *Diffusing capacity for carbon monoxide*
$\frac{DL_{CO}}{TLC}$ = *Diffusing capacity divided by total lung capacity*
D_m = *Diffusing capacity of pulmonary membrane*
Q_c = *Pulmonary capillary blood volume*
RV/TLC = *Residual volume divided by total lung capacity (hyperinflation index)*

Table 3. Pulmonary Function Means by Treatment and by Variable at 61 Months of Exposure (cont.)

	CA	R	I	SO_x	R + SO_x	I + SO_x	NO_L + NO_{2H}	NO_H + NO_{2L}	SD(E)[a]
MBC (liter/sec)	34.8	38.5	35.3	35.3	37.3	36.7	32.3	34.5	5.0
PF (liter/sec)	110	125	111	108	115	114	100	112	17
N_2 washout %	1.44	1.46	1.77	1.14	1.40	1.43	1.34	1.57	0.49
FRC (ml)	347	414	369	370	418	376	402	408	79
ERV (ml)	142	152	149	159	138	137	167	149	44
RV (ml)	232	267	220	212	280	239	235	258	56
VC (ml)	922	1,000	926	966	943	999	969	962	158
TLC (ml)	1,102	1,188	1,087	1,144	1,139	1,166	1,183	1,175	182
IC (ml)	727	775	719	773	734	790	782	767	132
N	18	11	10	9	10	11	11	11	

[a] Standard deviation for replicate animals (experimental error).

Key: *MBC* = *Maximum breathing capacity*
PF = *Peak flow*
N_2 washout % = *Percent rise in nitrogen between 5 and 7 times the dead space (nitrogen washout – index of uniformity of distribution)*
FRC = *Functional residual capacity*
ERV = *Expiratory reserve volume*
RV = *Residual volume*
VC = *Vital capacity*
TLC = *Total lung capacity*
IC = *Inspiratory capacity*
N = *Number of animals per treatment*

midprecordial P-waves of a dog exposed to oxides of sulfur were not collaborated by similar QRS-and-P-loop changes in the corresponding VCG, although LAD had been present on the previous VCG.

These P-wave and QRS-axis changes suggested right atrial and left ventricular abnormalities in depolarization, the causes of which were not apparent. Positive findings on post-exercise ECG's were distributed between two animals, one exposed to $R+SO_x$ and one to NO_L + NO_{2H}. All of the abnormalities noted were mild and subtle. Three animals, the exception seen in $R+SO_x$, demonstrated ventricular premature contractions. Although this type of arrhythmia after exercise may imply ischemia, the meaning of VPC's in dogs has not been well defined.

To summarize, the incidence of both static and exercise ECG abnormalities is 8.2% (6 of 73) vs. 0 in controls. This suggests air pollutants can be an etiologic agent inducing cardiac electrophysiologic damage.

There was also an association between air pollutant exposure and positive VCG abnormality in a dog exposed to clean air, that of LAD attributable to an acquired mitral stenosis. This could be discounted as was its ECG. The number of other dogs per treatment that developed, or consistently had, diagnoses of VCG abnormality were as follows: clean air, none; R, none; I, three; SO_x, two; $R+SO_x$, none; $I+SO_x$, one; NO_L + NO_{2H}, three; NO_H + NO_{2L}, three (Table 5). This incidence of 16.4% abnormalities in air pollutant-exposed dogs versus 0% in control dogs is striking. The VCG diagnoses of abnormality were not identical for dogs within

Table 4. Summary of the Electrocardiographic Data

	Incidence			
Treatment	Pre-Exercise	Post-Exercise	Period	Finding
CA	1/18	1/18	1, 2	LAD
I	1/10	0/10	1, 2	bradycardia
SO_x	1/19	2/9	2	LAD, precordia P-waves
$R+SO_x$	1/10	1/10	2	bradycardia
NO_L+NO_{2H}	0/11	1/11		VPC

3 of 73 = 4.1% resting
6 of 73 = 8.2% post-exercise

Table 5. Summary of Vectorcardiographic Data

Treatment	Incidence	Finding
CA	0/18	mitral stenosis
NO_H+NO_{2L}	3/11	
NO_L+NO_{2H}	3/11	
R	0/11	
I	3/10	RVH
$I+SO_x$	1/11	
$R+SO_x$	0/10	
SO_x	2/9	

12 of 73 = 16.4%

an air pollutant treatment group, except for irradiated auto exhaust where right ventricular hypertrophy, ranging from questionably present to mild, was consistently present.

Another study was designed to assess in each of the eight treatment groups the relative incidence of cardiac arrhythmias, which could represent air pollution-related cardiac pathophysiology. Electrocardiographic telemetry was performed for a 13½- to 15-hour diurnally identical period on each living dog (93) exposed for 4 years. An arrhythmia-monitoring system identified widened QRS complexes (W-QRS-C) and recorded them on a bar graph. Planimetry area of the bar graphs divided by time, in hours, resulted in a Widened-QRS-Complex Index (Table 6). The mean W-QRS-C indices were similar (deviation less than 8.6%) for dogs in clean air, auto exhaust, oxides of sulfur, high nitrogen dioxide and auto exhaust plus oxides of sulfur.

The $NO_H + NO_{2L}$ treatment mean was markedly but not statistically lower than the above treatments by approximately 72%. Dogs exposed to irradiated auto exhaust alone and with oxides of sulfur had mean W-QRS-C indices greater than controls by 31 and 42% respectively. Analysis of variance on pooled individual mean W-QRS-C indices of dogs exposed to irradiated auto exhaust and on their corresponding controls resulted in a significant probability of 0.057. The latter strongly suggests that exposure to irradiated auto exhaust may cause an elevated Widened-QRS-Complex Index.

A third study was an assessment of the effects of 4 years of exposure to air pollutants on blood viscosity changes resulting from alterations in blood cellular or extracellular composition. Measurements employing the Wells-Brookfield cone-plate microviscometer revealed no significant differences in blood viscosity among any of the eight treatment groups, despite significant elevations of carboxyhemoglobin in the four auto exhaust exposures (Tables 7 and 8). Exposed treatment mean methemoglobin ranged from 0.36 to 1.29%; control values were 0.33%. There were no statistical differences detected among the eight treatment means for methemoglobin concentration (Table 8).

Comment

Due to the ubiquitous nature of nitrogen and sulfur oxides and auto exhaust (per se and photochemically reacted) in the ambient air of urban communities, the chronic car-

Table 6. Mean Widened-QRS-Complex (W-QRS-C) Indices for Air Pollutant-Exposed Groups of Dogs

Treatment	W-QRS-C Index
	$\bar{x}$ ± S.E.
CA	3.4 ± 0.6
R	3.5 ± 0.7
I[a]	4.5 ± 0.6
SO_x	3.1 ± 0.9
$R + SO_x$	3.2 ± 0.7
$I + SO_x$[a]	4.9 ± 0.5
$NO_L + NO_{2H}$	3.4 ± 0.8
$NO_H + NO_{2L}$	2.4 ± 0.8

[a] For I and I + SOx, P = 0.057

Table 7. Mean Canine Blood Viscosity (Centipoise)

Treatment	Hct% $\bar{x}$ ± S.D.	23.0 (6 rpm) $\bar{x}$ ± S.D.	230.0 (60 rpm) $\bar{x}$ ± S.D.
CA	45 ± 5	8.5 ± 1.9	4.6 ± 0.8
R	48 ± 4	8.3 ± 2.0	5.0 ± 0.6
I	46 ± 5	7.9 ± 1.0	4.7 ± 0.5
SO_x	43 ± 5	7.6 ± 1.9	4.7 ± 0.5
$R + SO_x$	48 ± 3	8.2 ± 2.2	4.9 ± 0.6
$I + SO_x$	46 ± 4	8.7 ± 1.9	5.1 ± 0.8
$NO_L + NO_{2H}$	43 ± 4	7.3 ± 1.5	4.6 ± 0.5
$NO_H + NO_{2L}$	42 ± 5	7.2 ± 1.6	4.4 ± 0.5

Table 8. Average Carboxyhemoglobin and Methemoglobin Concentrations in Canine Blood (%)

Treatment	Carboxyhemoglobin	Methemoglobin
CA	1.95 ± 0.45[a]	0.33 ± 0.29[a]
R	10.56 ± 1.37	0.36 ± 0.42
I	9.30 ± 1.41	0.89 ± 0.74
SO_x	1.86 ± 1.04	1.29 ± 0.41
$R + SO_x$	10.50 ± 1.10	0.69 ± 0.87
$I + SO_x$	9.74 ± 1.26	0.71 ± 0.85
$NO_L + NO_{2H}$	1.82 ± 0.99	0.66 ± 0.86
$NO_H + NO_{2L}$	2.18 ± 0.61	0.98 ± 0.45

[a] Standard deviation

diopulmonary changes reported in this study as resulting from long-term, low-level exposure to such pollutants denote serious potential health hazards to the populace of certain communities. Findings provide new research avenues for epidemiologists, clinicians and toxicologists to pursue as they assess the health hazards of air pollution.

On the other hand, the experimental atmospheres did not produce readily detectable, dramatic toxic responses following an exposure duration of 5 years. A key question is, are 5-year inhalation exposures to ambient or near-ambient levels of air pollution of sufficient duration to demonstrate or predict suspect, insidious human diseases? Five-year toxicological investigations are rare due to their expense and the impatience of investigators, their administrative supervisors and funding entities. However, if one is to qualitatively and quantitatively generate animal model systems to duplicate or simulate human responses demonstrated or suggested in epidemiological studies, such long-term studies will be required. The more dramatic, non-allergenic responses to air pollution in humans occur after 40 to 60 years of exposure.

The results presented in this paper stem from studies performed 6 to 9 years ago. Since these studies were conducted, two new concepts of lung function have become very relevant in assessing pulmonary impairment resulting from exposure to airborne contaminants (17). The first relates to the importance of small airway function, both from the standpoint of site of action and as the earliest indicator of response. The second is concerned with the defense mechanism of the lung to protect the lung parenchyma against proteolytic enzymes released by cells and macrophages within the lung. Air pollutants, both particulate and gaseous, lead to an increase of these cells and macrophages in the lung in response to the deposition of particulates and irritation of the respiratory mucosa.

Chronic obstructive lung disease (COLD) has been increasing at an alarming rate as is evidenced by the number of deaths attributed to it (18). A 25% increase was noted from 1965 to 1966 alone (19). An important point with respect to COLD is that the site of the obstruction is in the small airways (20). The best test for evaluating small airway obstruction is the analysis of maximum expiratory flow-volume at small lung volumes of helium isoflow. None of the procedures of this analysis were employed on the dogs during the exposure phase of the study. Many of these procedures were making their debut at the termination of our role in this study.

Pulmonary function tests used to evaluate airway response would include: total expiratory resistance, peak expiratory flow, maximum breathing capacity and nitrogen washout. Those treatments associated with airway response are auto exhaust, auto exhaust plus SO_x, irradiated auto exhaust, NO_L + NO_{2H}, and SO_x. Those tests applicable to evaluating alveolar, parenchymal response would include: diffusing capacity, compliance and the lung volumes.

Treatments associated with alveolar, parenchymal response include auto exhaust, auto exhaust plus SO_x, NO_L + NO_{2H} and NO_H + NO_{2L}. One can only conclude that the pollutant atmospheres were not selective in affecting either airways or parenchyma but, in essence, affected both. Tests to evaluate small airway disease, however, were not available.

This study did demonstrate that exposure to air pollutants will produce the following aspects of lung disease: pulmonary hyperinflation, non-uniform distribution of inspired air, reduced diffusing capacity and elevated respiratory resistance. The cardiovascular responses indicative of disease include: cardiac arrhythmias, ventricular axis deviation, bradycardia and premature ventricular contractions.

Two important features of response to air pollutants were demonstrated in the study. First, exposure to the air pollutants resulted in a gradation of individual responses even within the same treatment. The incidence of individuals responding was the most sensitive means of detecting treatment effect. Secondly, the exposed individuals, at the pollutant concentrations investigated, demonstrated a decreased ability to perform vital functions and not an absolute failure of a subsystem component resulting in death or marked morbidity.

References

1. Swann, H.E., Jr., and O.J. Balchum. 1966. Biological effects of urban air pollution: IV. Effects of acute smog episodes on respiration of guinea pigs. Arch. Environ. Health 12: 698-704.
2. Wayne, L.G., and L.A. Chambers. 1968. Biological effects of urban pollution: V. A study of effects of Los Angeles atmosphere on laboratory rodents. Arch. Environ. Health 16: 871-885.
3. Emik, L.O., *et al.* 1971. Biological effects of urban air pollution: Riverside summary. Arch. Environ. Health 23: 335-342.

4. Hueter, F.G., *et al.* 1966. Biological effects of atmospheres contaminated by auto exhaust. Arch. Environ. Health 12: 553-560.
5. Murphy, S.D. 1964. A review of effects on animals of exposure to auto exhaust. J. Air Pollut. Contr. Assoc. 14: 303-308.
6. Clark, D.W., and B. MacMahon. 1967. Preventive medicine. Little, Brown and Co., Boston, pp. 729-732.
7. Ayres, S.M., and M.E. Buehler. 1970. The effects of urban air pollution on health. Clin. Pharmacol. Ther. 11: 337-371.
8. Ayres, S.M., *et al.* 1969. Systemic and myocardial hemodynamic responses to relatively small concentrations of carboxyhemoglobin (COHb). Arch. Environ. Health 18: 699-709.
9. Cohen, S.I., M. Deane, and R. Goldsmith, Jr. 1969. Carbon monoxide and survival from myocardial infarction. Arch. Environ. Health 19: 510-517.
10. Brinkman, R., *et al.* 1964. Radiomimetic toxicity of ozonized air. Lancet 1: 133-134.
11. Cascone, A., and R. Malusardi. 1968. Myocardial infarct of allergic pathogenesis: Description of a case. Minerva Cardioangiol. 16: 1161-1166.
12. Vaughn, T.R., *et al.* 1969. Long-term exposure to low levels of air pollutants: Effects on pulmonary function in the beagle. Arch. Environ. Health 19: 45-50.
13. Lewis, T.R., *et al.* 1974. Long-term exposure to auto exhaust and other pollutant mixtures. Arch. Environ. Health 29: 102-106.
14. Bloch, W.N., Jr., *et al.* 1972. Cardiovascular status of female beagles exposed to air pollutants. Arch. Environ. Health 24: 342-353.
15. Bloch, W.N., Jr., *et al.* 1973. Blood rheology of dogs chronically exposed to air pollutants. Tox. Appl. Pharm. 25: 576-581.
16. Bloch, W.N., Jr., *et al.* 1970. Electrocardiographic telemetry with arrhythmia recording on dogs chronically exposed to air pollutants. Unpublished, pp. 1-11.
17. Bates, D.V. 1972. Air pollutants and the human lung. Am. Rev. Resp. Dis. 105: 1-13.
18. McFadden, E.R., and D.A. Linden. 1972. A reduction in maximum mid-expiratory flow rate. Am. J. Med. 52: 725-737.
19. USPHS review. 1968. Health consequences of smoking, 1968 supplement, Washington, D.C.
20. Hogg, J.C., *et al.* 1968. Site and nature of airway obstruction in chronic obstructive lung disease. N. Engl. J. Med. 278: 1355-1360.

Asilomar Conference Discussion

Lewis: . . . And there's no reason to believe that you can't have an impaired dog even though she is in clean air. She was a small dog, as I remember, and could have been impaired somewhat genetically, or during the testing procedure there were possibilities of aspiration, pneumonias, and things of this nature, since aspiration can occur while recovering from the anesthesia, etc. So you can induce artefacts or pulmonary disease just in your methodology.

Thurlbeck: Why are you interested in lower compliance rather than higher compliance?

Lewis: You mean in terms of specific compliance? We were looking for fibrosis and things of that nature.

Thurlbeck: Not for hypercompliance?

Lewis: We didn't have any. Hypercompliance usually precedes chronic obstructive lung disease, and in our studies, when we perform pulmonary function tests sequentially and get hypercompliance, it's indicative that we have to continue the exposure; that the ultimate result is going to be chronic obstructive lung disease, small airway involvement, etc. But we do look at it. However, the clinical significance of hypercompliance is a little difficult to interpret. It may relate to emphysema.

Albert: You didn't use each animal as a control to look at sequential changes, did you?

Lewis: I thought I brought this out but I'll restate it. We didn't have baseline information to do that.

Albert: Well, you did it at 18 and 36 months.

Lewis: Well yes, sequentially in that sense, however, I don't know what that would have told you.

Albert: I'd just look at the data.

Lewis: Well, I'll tell you, other factors are involved in measurement of pulmonary function; the same values in the same dogs aren't repeated from day to day. In itself, the same test can be quantitatively different from one testing period to another.

Nettesheim: You said before that the best control is the animal itself.

Lewis: That's right. That's the best control we have, but we don't have absolute control.

Busch: The pre-exposure data would have to have been taken when the animals were puppies?

Lewis: Four to six months of age.

Busch: So there probably wouldn't have been good correlations between the later values and the very young.

Lewis: I think that from our experience in this and other studies, the regression lines differ. Compliance would go up; resistance would go down. That type of phenomenon is associated with aging, particularly from puppyhood into maturity.

Gillespie: The peak flows from the $NO_L + NO_{2H}$ group were greater than the controls. Is that right?

Lewis: No, they were lower. What we're talking about is peak flows, above 80 liters per minute. The absolute criterion is what we used.

Gillespie: That was then measured during expiration?

Lewis: Maximum expiration, yes. So, the most dramatic effects obviously are the NO_2-high in terms of pulmonary impairment. There are other effects which appear to be physiologically significant and interpretable in different ways.

MacEwen: If you use the SO_x groups as part of your control for comparison in the first set up at the top, why don't you do it in the bottom when you're comparing the R + SO_x? For your RV/TLC.

Lewis: The comparison is the summation of two groups up at the top, and down at the bottom one group is merely compared against another group. Such contrasts reflect differing components of factorial design. This result was the only one found in terms of one treatment versus another treatment.

Hueter: The question is, is it the R or the SO_x that is causing the effect? You've got the R+SO_x together.

Lewis: I don't know, Dr. Hueter, they are working together and producing an additive effect. This was 6 years ago. This was the most dramatic comparison observed and it appeared to be an additive effect. I think we have some material which supports this in another fashion. RV/TLC . . . it's residual volume data where three treatments were significantly different from the irradiated, and they were R and R+SO_x, and NO_2-low treatments. Let me look back here, if I can pick it up very rapidly. "Dogs receiving auto exhaust plus oxides of sulfur and those receiving NO_2-low demonstrated elevated residual volumes: 280, 266, and 258 ml versus 232." These results were from the analysis of variance. So there appears to be an association . . . the one that's common to all three treatments — the auto exhaust, the auto exhaust + SO_x, and NO_2-low — is the concentration of nitrous oxide.

Busch: In the RV/TLC, how many in the raw were outside the range?

Lewis: I don't have those data. That was the smallest sample size group of the eight treatments. We only had eight at that point in time, and that made it difficult in an individual comparison because we were losing degrees of freedom, but the incidence data were helpful.

Busch: In the 9 of 21 in the first line, could the figures for I and I+SO_x be pooled? Were they comparable?

Lewis: They were comparable.

Thurlbeck: Is the same animal the exception; for example in R+SO_x?

Lewis: Not necessarily, and unfortunately I don't have the raw data to differentiate between them anymore.

Albert: What magnitude of bradycardia are you talking about?

Lewis: I'll have to look that up for you later to try to follow in the time frame. Vectorcardiograms (Table 5). . . . These are incidence data once again. Dr. Bloch seemed to feel that the one control cardiac incidence in the 1/18 was due to the diagnostic congenital mitral stenosis and could be scientifically excluded. Thus, the respective incidence in each of the treatments was: 3/11 in the NO_2-low, 3/11 in the NO_2-high, 0 in the raw, 3/10 in the irradiated, 1/11 in the $I+SO_x$, 0 in the $R+SO_x$, 2/9 in the SO_x treatment, for a total of 12/73 in the seven exposure groups versus 0/18 or an incidence of 16%.

MacEwen: What happened to the rest of the dogs? Why 73? Weren't there 90-some at this point? What happened to the other 20 dogs? Were the data not used?

Lewis: Add the controls into this and you come up with 91 at this point in time.

MacEwen: Well, the controls were listed above.

Lewis: Right, but there's 0/18 versus 12/73. This was the contrast that was made. We were not able to pick out, say, any one of the seven exposure treatments that induced more cardiovascular abnormalities than any other, but air pollutants *per se* had this incidence versus the clean air dogs.

Albert: The RVH is right ventricular hypertrophy?

Lewis: Yes, and the reason I put that on the slide was that the only treatment that appeared to have a similar abnormality within it was the irradiated treatment, and all three of these dogs had evidence of right ventricular hypertrophy.

Albert: Was that confirmed at autopsy?

Lewis: By measurement and weight of the total heart right and left ventricles. The autopsies were done several years later by the Davis group.

Thurlbeck: What about mitral stenosis?

Lewis: It was confirmed by other types of tests, but not by autopsy tests.

Orthoefer: The right ventricle and left ventricle plus septum were weighed separately. If the three of ten animals listed were outstanding, they would have shown up. I can't say without knowing what animals, but according to a series of tests that were run, there was no statistical significance as far as treatment is concerned in the organ weights.

Lewis: Your answer doesn't exactly answer his question. Dr. Albert wants to know whether these particular dogs had right ventricular hypertrophy.

McLees: Did they have wedge pressure or anything like that done?

Lewis: Yes.

McLees: Were they abnormal?

Lewis: There was one abnormal wedge pressure; the other two dogs did not have abnormal wedge pressures.

McLees: The problem that is giving me and other people trouble is that the electrocardiogram is probably the least specific way of measuring RVH. If you have data to corroborate it, that's one thing, increased pulmonary vascular resistance or pulmonary artery pressure, something like that, or autopsy data. What criteria were used?

Lewis: ECG, VCG, and pulmonary cardiograms. The problem that I have is that I left EPA, and then Dr. Bloch who was a 2-year PHS Commission Corps Officer left, and he took the data with him to write the paper. As a result, I think we've lost the identity of individual values. We have only the summary statistics. I don't have the data, and I don't know whether they could be retrieved. I've lost track of Dr. Bloch, and do not have his present address. These studies were conducted 6 or 7 years ago.

Orthoefer: Wouldn't right ventricular hypertrophy show up as an increased heart weight as compared to the total body weight?

Dungworth: I think the point they're making, though, is when you had it analyzed, the data were lumped to see if there were differences between any treatment groups and controls. The question is whether for those three specific dogs there would be a significantly increased weight of the right ventricle or whole heart.

Orthoefer: If those three dogs could be singled out, then you could have it done.

Dungworth: Then you have the raw data.

Albert: Why can't they be singled out?

Lewis: We have to know the individual identity of each of the three dogs. All I have is the summary data, and Dr. Bloch had the raw data, when you're a physician moving from place to place, I would say this might be the first thing you threw out.

Orthoefer: By inspection, we could look at three dogs in that particular group and say, these three dogs look like they had high right ventricular hypertrophy.

Busch: I don't think you associate these effects with any particular component anyway. It doesn't seem to be associated with NO_2 because it doesn't appear in the raw exhaust, which has NO_2. It isn't associated with auto exhaust *per se*.

Lewis: We didn't make any association. We just said that if you were exposed to air pollutants you had this incidence compared to our filtered clean air animals.

Albert: Yes, but it would be nice to be able to say that the animal that had the severe morphologic damage of the lung showed the severest abnormalities in pulmonary function and they were the ones that had the right ventricular hypertrophy, etc.

Lewis: I couldn't concur with you more. This is one of the problems of running a chronic study and moving facilities and people.

. . . I want to point out the word average. When Dr. Bloch did this, we had a 16-hour exposure to the treatment atmospheres. He took the carboxyhemoglobin values every 6 hours, 40 determinations and averaged these. So these are not peak values. This is how the values were derived, and these values were what one would expect. Animals exposed to auto exhaust had higher carboxyhemoglobin. That's the only significant figure here. We also did a methemoglobin. This was an average of the methemoglobin prior to the initiation of the exposure and the methemoglobin at midnight, which was 16 hours later. These are the values here, once again slightly elevated in any group other than controls but not statistically significant.

Hueter: Why was it that way?

Lewis: I don't know, maybe it's an artefact. I don't have any reason. It could be the way the numbers fell out or it could be a sulfhydryl-type reaction. I have no physiological explanation for it.

Busch: Trent seems to have made a point about the incidence approach being more sensitive than the analysis of variance. We never recommend taking means of animals that have been affected along with animals that have not. The only time it is appropriate to compare means is when the variance remains the same, the standard deviation remains the same for the treated as it does for the control group, so you can see that the animals respond similarly but retained their similarity among themselves. In the case where there are some animals that respond at a given point in time and others that do not, then what you've done is what should be done. As a matter of fact, I suggested that yesterday for the biochemical data; to take an approach like that instead of comparing the means.

Lewis: You have to have a handle on each dog as an individual. You can do it by covariance, which is a convenient means, or you can do it by incidence data, but to use a variable like body weight or just to compare treatments where you've got a big dog who has a high TLC and another smaller dog with a low TLC, you can have the same variability within treatments, but you increase the variability in your error terms because one dog has 700 and another has 1000.

Busch: Yes, but you need a high correlation between the pre- and post-values, and I don't believe there would have been a good correlation here because of the long time lapse and the change in the aging effect.

7. REVIEW OF THE CARDIOVASCULAR AND PULMONARY FUNCTION STUDIES ON BEAGLES EXPOSED FOR 68 MONTHS TO AUTO EXHAUST AND OTHER AIR POLLUTANTS

J. R. Gillespie

Summary

This review includes the results of physiology studies on the cardiovascular and pulmonary systems of groups of beagles exposed for 68 months to low levels of auto exhaust, SO_x, NO, NO_2 and combinations of auto exhaust and SO_x. Cardiovascular and pulmonary function measures were made on each beagle at intervals during and following the exposure period.

There were a few individuals in each group (including one in the control group) with some evidence of cardiovascular dysfunction. There is no substantial evidence that the cardiovascular dysfunction recorded was caused by air pollution exposure. Some of the abnormalities were most likely congenital.

Periodically during the exposure period and 2 years after the termination of exposures (2YR), investigators in Cincinnati or at UC-Davis measured the beagles' arterial blood gases, lung volumes, chest wall and lung compliance (C_L, C_{cw}), dead space (V_D), frequency dependent compliance (C_{dyn}/f), carbon-monoxide diffusing capacity ($D_{L_{CO}}$), and respiratory system, chest wall, and pulmonary resistance (R_{rs}, R_{cw}, R_{pul}). We compared the 2YR values made at UC-Davis with those from studies during and immediately after (TE) the 68-month exposure made by another research group in Cincinnati. The 2YR values of the control group were similar to their TE values and to those of other healthy beagles studied in the UC-Davis laboratory. All exposure groups had pulmonary function and structural differences from the control group, and these groups, but not the controls, had more functional abnormalities at 2YR than at TE.

In general, auto-exhaust (R and I) exposure appeared to injure airways and parenchyma, whereas SO_x, NO, and NO_2 caused injury to parenchyma. Combinations of auto exhaust and SO_x did not appear to augment specific functional losses caused by each single species of pollutant. The functional abnormalities correlate well in most instances with structural changes in the exposure groups. Our studies support the hypotheses that: 1) exposure to low levels of specific air pollutants produces pulmonary injury and loss of pulmonary function, and 2) the functional loss continues following termination of the exposure.

Introduction

A multidisciplinary study of the effects of exposure to auto exhaust and other pollutant mixtures upon the cardiovascular and pulmonary systems of beagles was undertaken beginning September 1965. It was designed to enable study of the effects upon the pulmonary system of long-term (68 months), low-level exposure to air pollutants. The exposures were in the chambers at the Environmental Research Center, Cincinnati (1, 2).

Cardiovascular and pulmonary function studies were done periodically on the beagles by the investigators in Cincinnati during exposure and immediately after termination of exposure.

About 6 months after the termination of exposure, 86 of the original 104 beagles were transported to Davis, where we studied the pulmonary function and cardiovascular function of 71 of them about 2 years and 3 years after the termination of exposure. Those not studied had pulmonary lobectomies. After these studies were complete, the animals were killed and the morphology of their lungs was studied. Our aim was to evaluate the cardiovascular and pulmonary function of these beagles for evidence of residual effects of the air pollutants 2 years after exposure. This paper presents our findings and correlates them with earlier measurements upon these beagles during the exposure period.

Materials and Methods

Animal exposure: The air-pollutant exposure protocol and technique was described in detail by Hinners and co-workers (1) and Hueter and co-workers (3). The exposure facility was located at the National Environmental Research Center, Cincinnati. In brief, 104 female beagle dogs were assigned to stainless steel and glass exposure chambers which contained four dogs each. Beginning at 6 months of age a control group (group 1) of 19 dogs was exposed to filtered, temperature- and humidity-conditioned clean air, and the remainder of the dogs breathed pollutants for part of each day. Those in the exposure chambers were divided into seven treatment groups and exposed 16 hours each day to one of the following pollutants or mixtures for 68 months: group 2, raw auto exhaust (R); 3, irradiated auto exhaust (I); 4, SO_x; 5, R + SO_x; 6, I + SO_x; 7, NO_2 (high conc.) + NO (low conc.); 8, NO (high conc.) + NO_2 (low conc.) (see Table 4 in Chapter 2).

Cardiovascular function, Cincinnati: Bloch and co-workers (4) described the methods they employed to evaluate the cardiovascular function of the beagles at age 4½ and 5 years during the period of exposure. In brief, they recorded electrocardiograms (ECG) at rest and after swimming exercise and devised a grading system to evaluate the graphs. They recorded vectorcardiograms at rest on all dogs and phonocardiograms on those with some evidence of cardiac dysfunction. They measured venous, right ventricular, pulmonary and systemic arterial pressures on all the beagles under pentobarbital anaesthesia.

Cardiovascular function, UC-Davis: Seventy-one beagles ranging in age from 8¼ to 8½ years were studied after being fasted for 24 hours. They weighed between 7.25 kg and 12.47 kg. They had previously been divided into eight treatment groups as shown in Table 4 of Chapter 2. We studied them 36 months after the termination of exposure.

They were anaesthetized intravenously with sodium pentobarbital (50 mg/ml) and intubated with a 9-mm endotracheal tube. A 5-mm Swan-Ganz flow-directed right heart catheter was introduced into the femoral vein and passed into the right ventricle, pulmonary artery, and pulmonary wedge pressures. A 7-F Goodall-Lubin standard wall catheter was placed in the femoral artery and advanced approximately 15 cm into the aorta. Systolic, diastolic and mean arterial pressures were recorded. The recording system was calibrated daily against a mercury manometer, and the transducers were leveled with the base of the heart.

Cardiac outputs were measured using the indicator-dilution method in which we used indocyanine green dye. We injected 1.25 mg (approximately 0.13 mg/kg) dye into the pulmonary artery followed immediately by a 5-ml flush of heparinized saline. Arterial blood was pulled through a cardiac output computer cuvette by means of a constant flow syringe puller. Cardiac output was recorded digitally on the cardiac output computer and the dye dilution curve was simultaneously recorded. The cardiac output computer was calibrated electrically before each measurement and this electrical signal was checked against standard dilutions of indocyanine green in whole blood.

From the blood pressures and cardiac outputs, the following information was obtained: heart rate (beats/min), stroke volume (ml), cardiac index (L/min/kg$^{3/4}$), total peripheral resistance (dynes/sec/cm^{-5}), total pulmonary resistance (dynes/sec/cm^{-5}) and left ventricular work (L/mm Hg/min^{-1}).

Pulmonary function, Cincinnati: Vaughn and co-workers (5) and Lewis and co-workers (6) described in detail their methods to study pulmonary function after 18, 36 and 61 months of exposure. In brief they measured lung compliance, subdivisions of long volume, pulmonary resistance, single-breath, carbon-monoxide diffusing capacity, maximum breathing capacity and peak expiratory flow. From these measurements they calculated ratios for compliance/lung volume and diffusing capacity/lung volume.

Pulmonary function, UC-Davis: The pulmonary function techniques we employed in this study have been reported by us in detail (7, 8, 9), and only a brief account will be given here for each.

The beagles were delivered to the laboratory, weighed, and given a physical examination. The pulse and respiratory rate were recorded, as were any comments of special interest resulting from the physical examination. Chest measurements (width, depth, and length) were made and a venous sample was taken for packed cell volume (PCV) and hemoglobin (Hb) analysis.

The beagles were anaesthetized intravenously with thiamylol sodium[1], 8 mg/kg, and intubated with the largest possible diameter endotracheal tube (8-9 mm I.D.). An esophageal balloon[2] 10 cm long (containing 0.5 ml air) on PE 200 tubing was placed in the esophagus and attached to a pressure transducer[3] for measuring pleural pressure. The dogs were ventilated during most of the study with a constant volume ventilator.[4] The ventilatory volume and rate were adjusted to give end-tidal CO_2 concentrations of approximately 5%, measured by an infrared CO_2 analyzer.[5] Deep breaths at 5-minute intervals to a transpulmonary pressure (P_{TP}) of 30 cm H_2O prevented the fall in lung compliance reported in anaesthetized dogs (10,11).

We used a volume displacement plethysmograph[6] specifically designed for dogs connected to a wedge spirometer[7] to measure slow changes in lung volume. The plethysmograph was converted to a pressure box to record fast lung volume changes using a highly sensitive

[1] Surital, Parke-Davis
[2] Anode Rubber Plating Co., no. UC-516
[3] Statham, PM131-TC
[4] Harvard, model 614
[5] Beckman, model LB1
[6] K-plastix, San Francisco
[7] Med-Science Electronics, model 270

pressure transducer[8]. We have previously described the performance characteristics of our measuring apparatus (8). All data were recorded by an 8-channel oscilloscopic recording system[9].

Soon after giving anaesthesia and prior to mechanically ventilating the beagles, we collected data to calculate physiological dead space volume (V_D) and dead space/tidal volume (V_D/V_T) ratios. We collected expiratory gas in a bag in a rigid box. Spontaneous tidal volume and breathing frequency were recorded by a wedge spirometer attached to the rigid box. At the midpoint of the 3-minute expired gas collection, we anaerobically collected an arterial blood sample from the femoral artery. We measured[10] arterial blood P_{O_2}, P_{CO_2}, and pH immediately after collection, and corrected our values to the dog's deep rectal temperature. The mixed expired sample was analyzed for O_2 and CO_2 concentration with a Scholander gas analyzer[11]. We used the conventional formula to calculate V_D/V_T (12).

We measured resistances of the respiratory system using the method of Mead and Wittenberger (13). Sinusoidal flow oscillations were generated by a speaker system attached to the endotracheal tube. Following a deep breath, the dog's lungs were allowed to return to resting, end-expired volume (FRC), and we oscillated the airway at 3 to 5 Hz. We recorded data for total pulmonary resistance (R_{pul}), total respiratory resistance (R_{rs}), and chest wall resistance (R_{cw}) by recording transpulmonary, airway, and pleural pressure, respectively, versus flow on an x-y oscilloscope. Flow rates were measured with a pneumotachograph[12] attached to a pressure transducer[13]. Endotracheal tube resistances were subtracted to determine R_{pul}, R_{rs}, and R_{cw}.

We measured pulmonary diffusing capacity with the single-breath carbon-monoxide test ($D_{L_{CO}}$) (7, 9, 14). We rapidly injected a volume of test gas (0.3% CO, 0.5% Ne, balance air) equivalent to the dogs inspiratory capacity (IC) into the dog's lungs, allowed a 10-second breath-hold, then collected an alveolar sample for analysis[14]. We calculated $D_{L_{CO}}$ using the formula of Ogilvie *et al.* (15). We repeated these measurements after each beagle had breathed oxygen-rich gas for three breaths and 10 to 15 minutes. From these measurements we calculated pulmonary capillary blood volume by the technique of Roughton and Forster (16).

We recorded quasistatic lung and chest wall pressure-volume curves using techniques reported earlier by this laboratory (7, 8). We inflated the dog's lungs until P_{TP} = 30 cm H_2O, and allowed them to relax to FRC. Then we recorded volume on the y-axis and P_{PT} on the x-axis as we slowly inflated the lungs to P_{TP}=30 cm H_2O, slowly deflated them to P_{PT} = 15 cm H_2O, and allowed passive reinflation to FRC. The pressure-volume trace was nearly horizontal from P_{TP} = 10 to -15, and this volume was taken to be residual volume (RV).

[8] Statham, PM5
[9] Electronics for Medicine, model DR8
[10] Blood Gas Tension Analyzer, model 113, Instrumentation Laboratories
[11] Scientific Instruments
[12] Sims Instruments, Fleish model 1 (60 l/min)
[13] Statham, PM5
[14] Micro-Tek, Tracor, Respiratory Gas Analyzer, model 150

Chest wall volume-pressure curves were recorded using the same maneuvers with volume plotted against pressure across the chest wall (P_{cw}).

We measured end-expiratory lung volume (FRC) following an inflation-relaxation maneuver with two techniques: 1) the nitrogen equilibration technique, FRC_{N_2} (7, 8), and 2) the Boyle's law technique, FRC_{BL} (7, 8). In the first, following a lung-inflation–passive-deflation maneuver, we ventilated the dog manually 15 times with a volume of O_2 equivalent to its IC. This was done by using a syringe to move the oxygen in and out of the dog's lungs. The fraction of N_2 in the syringe at the end of the test was analyzed[15], and the FRC_{N_2} calculated (7).

In the second technique, done immediately prior to the first, we measured the plethysmograph volume and airway pressure changes while the dog, beginning at FRC, breathed spontaneously against an obstructed airway. We calculated FRC_{BL} from slope of the volume-pressure record (8).

We measured dynamic compliance (C_{dyn}) at 5, 10, 20, 30, 40, and 50 breaths/minute. We compared the slopes of the plot of the C_{dyn} against the frequency for each group. The greater the slope, the greater frequency dependency of lung compliance.

We compared the pulmonary function values of these beagles to those of a group of healthy beagles studied in our laboratory using the same techniques (7, 8, 9) and, where techniques permitted, to their own values measured 2 years earlier in Cincinnati (6).

Pulmonary function values for the 8 groups were evaluated statistically with Duncan's multiple range test. Changes in individual's values within each group between termination of exposure (TE) and 2 years after termination of exposure (2YR) were evaluated with the paired Student-t test. We accepted the 0.05 probability level as being significant when comparing values from the different groups.

Results

Cardiovascular function, Cincinnati: The cardiovascular studies done during the course of exposure were described by Bloch and co-workers in three papers (4, 17, 18). The results of their studies are summarized in Tables 1 through 8.

No specific abnormal function can be attributed to air pollution exposure from their studies. There were no trends within groups or within individuals to suggest progressive cardiovascular dysfunction in the exposed beagles. There were individual dogs with cardiac dysfunction in various groups (including an individual in the control group). Table 8 shows the hematocrit, carboxyhemoglobin and methemoglobin values recorded by Bloch and co-workers (4) during the period of exposure.

Cardiovascular function, UC-Davis: There was no difference in the body weight (range of means 9.2 to 10.2 kg), PCV (range of means 37.3 to 40.9%) or Hb (range of means 14.2 to 15.8 gm%) of the eight groups of beagles. The results from the cardiovascular measurements at UC-Davis 3 years after termination of exposure are in Tables 9 through 11. There was no

---

[15] Nitrogen Analyzer, Med-Science, model 105

Table 1. Grading System for Electrocardiograms[a]

ECG grade	Meaning[b]	Gradation
0	Within normal limits	. . .
I-A	Abnormal and nonspecific (even for heart disease), but questionable abnormality	. . .
I-B	Abnormal and nonspecific (even for heart disease), not questionable abnormality	. . .
II	Abnormal and probable heart disease of nonspecific kind	. . .
III	Abnormal and probable heart disease	. . .
IV-1	Abnormal and possibly compatible with ________________heart disease	$\frac{1+}{4+}$
IV-2	Abnormal and definitely compatible with ________________heart disease	$\frac{2+}{4+}$
IV-3	Abnormal and suggestive of ________________ heart disease (more than compatible, though not yet conclusive)	$\frac{3+}{4+}$
IV-4	Abnormal and diagnostic of ________________ heart disease (conclusive)	4+ 4+

[a] Bloch *et al.* (4)

[b] The type or types of heart disease is inserted in blank spaces in sentences IV-1 through IV-4.

Table 2. Some ECG Interpretation Grading Criteria[a]

ECG grade assigned	Associated abnormalities[b]
0	(a) P-wave notched in V1 (b) ST-segment elevated or depressed less than 0.1 mv (c) QRS/T-angle greater than 90° in one plane only
I-A	(a) Bradycardia or tachycardia (b) ST-segment elevated 0.1 to 0.15 mv (c) QRS/T-angle greater than 90° in frontal and horizontal planes (d) Increased right or left precordial R-waves (e) Increased right or left precordial S-waves (f) Combinations of (b) and (c)
I-B	(a) P-wave 0.3 to 0.35 mv (b) T-wave inverted or peaked in V1-6 (c) ST-segment elevated 0.15 mv or more (d) ST-segment depressed 0.1 mv or more
II	(a) P-wave 0.35 mv or more (b) P-wave greater than 0.04 sec (c) Mild arrhythmia (other than sinus arrhythmia) (d) Sinus pauses of 1.8 sec or more which are at least double the average R-R interval (e) Right- or left-axis deviation
III	(a) Severe arrhythmia[c] (b) Combinations of I-A, I-B, II

[a] Bloch *et al.* (4)

[b] This list includes only most commonly encountered abnormalities which are not unequivocally associated with definite cardiac pathology.

[c] This could also be designated as grade IV in some cases.

Table 3a. ECG, VCG, and Other Diagnoses, by Sampling Period[a]

Treatment	Static ECGs					
	1a	1b	1c	2a	2b	2c
Clean air	II	II	II	II	II	II
	I-A	0	I-A	I-A	I-B	I-A
R	I-A	0	I-B	0	0	I-A
I	0	I-A	0	0	0	0
	II	0	0	0	0	0
	I-A	I-A	I-A	I-A	I-A	I-A
	0	0	0	I-A	0	0
	I-B	0	I-B	X	X	X
SO_x	I-B	I-A	0	I-A	0	I-A
	0	0	—	0	0	0
	0	I-A	II	II	II	III
	0	0	0	0	0	0
R + SO_x	0	0	0	I-A	0	—
	—	—	0	0	I-B	I-B
	0	0	0	I-A	0	I-A
	0	0	0	I-A	I-A	I-A
I + SO_x	0	0	0	I-A	I-A	I-A
NO_L + NO_{2H}	I-A	0	0	0	0	I-B
	II	I-A	0	I-A	I-B	I-B
	III	0	0	0	0	0
	0	0	0	0	0	0
	0	0	0	I-B	0	1
NO_H + NO_{2L}	I-A	0	0	0	0	0
	0	0	0	I-A	0	0
	0	0	0	I-A	0	0

[a] Bloch *et al.* (4)

Table 3b. ECG, VCG, and Other Diagnoses, by Sampling Period[a]

Treatment	ECG rating[c]	Vectorcardiograms[b]	
		1	2
Clean air	S	LAD	LAD
	D	WNL	WNL
R	S	~WNL	WNL
I	. . .	WNL	Mild RVH
	. . .	~WNL	? Mild RVH
	S	~WNL	WNL
	. . .	WNL	? Mild RVH
	D	WNL	X
SO_x	S	? Mild RVH	Possible Mild RVH
	. . .	~WNL	WNL
	S	LAD	WNL
	. . .	Possible mild RVH	WNL
R + SO_x	. . .	? Mild RVH	? Mild RVH
	D	LVH vs VCD	Mild to moderate LVH
	S	WNL	WNL
	S	WNL	? Postinfarct
I + SO_x	D	WNL	WNL vs IVCD
NO_L + NO_{2H}	. . .	WNL	Mild LVH
	D	~LVH, mild	Moderate LVH
	. . .	~variant WNL	RVH (? mild)
	. . .	WNL	WNL
	. . .	WNL vs mild RVH	WNL
NO_H + NO_{2L}	. . .	~WNL	Probable old posterior infarct
	. . .	WNL vs infarct vs fibrosis	~mild RVH
	. . .	WNL	LAD

[a] Bloch *et al.* (4)

[b] Within normal limits, WNL; right ventricular hypertrophy, RVH; mean systemic arterial hypertension, SAH; diastolic systemic arterial hypertension, DSAH; ventricular conduction defect, VCD; left ventricular hypertrophy, LVH; intraventricular conduction defect, IVCD; left axis deviation, LAD; animal dead, X.

[c] Same ECG abnormality throughout each period, S; differing ECG abnormality within same period, D.

Table 3c. ECG, VCG, and Other Diagnoses, by Sampling Period[a], [b]

Treatment	Postexercise ECGs, sampling period 2	Catheterization diagnoses	Phonocardiographic diagnoses
Clean air	...	...	Mitral insufficiency
	...	...	
R	...	...	...
I	...	SAH	...
	...	...	...
	...	...	...
	...	...	...
	...	...	...
SO_x	...	...	...
	II	...	...
	...	...	...
	II	...	...
R + SO_x	...	...	...
	I-A(+)	...	...
	...	...	Mitral stenosis
	...	...	...
I + SO_x	...	...	...
NO_L + NO_{2H}	...	...	...
	...	...	...
	...	Pulmonic stenosis	...
	II	...	...
	...	SAH & DSAH	...
NO_H + NO_{2L}	...	...	...
	...	...	...
	...	...	...

[a] Bloch *et al.* (4)

[b] Mean systemic arterial hypertension, SAH; diastolic systemic arterial hypertension, DSAH.

Table 4. Selected Blood Pressures (mm Hg, ± 1 SD)[a]

Treatment	Pulmonary wedge	Individual pulmonary wedge	Mean pulmonary artery	Systolic pulmonary artery	Individual systolic pulmonary artery[b]	Mean blood pressure	Individual blood pressure
Clean air	2.43 ± 2.56	8.50[c]	17.84 ± 5.14	26.72 ± 6.56	40.5	122.4 ± 15.2	
R	2.68 ± 2.57		19.91 ± 4.54	28.91 ± 5.34		130.9 ± 10.6	
I	2.23 ± 1.30		17.65 ± 3.72	27.15 ± 6.07		120.9 ± 16.9	157.50
SO_x	2.33 ± 2.71		16.56 ± 2.75	25.78 ± 3.87		122.3 ± 20.2	86.25
R + SO_x	3.67 ± 4.17	13.25	19.82 ± 3.75	29.05 ± 4.39		124.0 ± 13.3	
I + SO_x	1.75 ± 2.74	-4.75[d]	17.50 ± 5.79	27.91 ± 10.53	50.0	123.8 ± 14.1	
NO_L + NO_{2H}	1.05 ± 1.64		18.28[c] ± 3.36	26.70[c] ± 3.44		124.8 ± 19.2	163.25
NO_H + NO_{2L}	1.22 ± 1.92		17.07 ± 3.56	27.05 ± 6.06	42.0	121.8 ± 12.8	

[a] Bloch *et al.* (4)

[b] Significant ($P<0.05$).

[c] Data from ten animals.

[d] Data from different animals in same treatment.

Table 5. Phonocardiographic and Physical Abnormalities[a]

Treatment	Findings on individual dogs	Diagnosis
Clean air	Mitral area: early systolic decrescendo murmur	Mitral insufficiency
R	. . .	. . .
I	. . .	. . .
SO_x	. . .	. . .
R + SO_x	Mitral area: mild to late diastolic murmur with presystolic accentuation	Mitral stenosis
I + SO_x	. . .	. . .
NO_L + NO_{2H}	Unable to pass catheter through pulmonary valve	Pulmonary stenosis
NO_H + NO_{2L}	. . .	. . .

[a] Bloch *et al.* (4)

Table 6. Dog Illnesses During Observation Period[a]

Diagnosis	Sampling period	Treatment	Findings
Probable myocardial infarction	2	R + SO_x	Dog weak, eyes shut; cardiovascular, S_1, S_2 within normal limits without murmur; ECG, ST: ↑1.5 mm in V_1, 1 mm in V_2, T-waves inverted and peaked, $V_{1\text{-}6}$ QRS/T-angle > 90° in horizontal plane only; these changes were not present on static ECGs from previous sampling period; ECG 5 days later, changes persisted with ST: ↑ 2 mm in V_1, 1.5 mm in V_2; QRS abnormally slurred, $V_{5\text{-}6}$; T-waves notched, 2,3, aVL, aVF; animal gradually recovered
Myocardial infarction	After 2	I + SO_x	Dog weak, moves little; faint S_1, S_2; ECG, pathological Q-waves, 1, aVL, V_5, V_6, V_{10}; T-waves inverted and slightly coved, 1, aVL; VCG decreased anterior forces, terminal QRS-delay, QRS-loop open; enzymes, LDH, and SGOT statistically significantly elevated ($P<.05$); ECGs 3 and 7 days later, pathological Q-waves decreased in size; T-waves reverted to upright; animal gradually recovered

[a]Bloch *et al.* (4)

Table 7. Dog Deaths During Observation Period[a, b]

Sampling period	Treatment	ECG premortem	Pathological heart findings	Likely cause of death
1	$NO_L + NO_{2H}$	6 days prior, compatible with schemia	Gross, not remarkable	Trauma
1	I	. . .	Gross, not remarkable	Trauma
1	I	. . .	Gross, not remarkable	Trauma
1	Clean air	. . .	Gross, not remarkable	Trauma
2	SO_x	Compatible with myocardial infarction	Histological, not remarkable	Trauma and probable myocardial infarction
2	SO_x	. . .	Gross, heart muscles replaced by probable connective tissue	Failure to recover from anesthesia, chronic neurologic disease suspected
After 2	$NO_H + NO_{2L}$	WNL (6 leads)	Gross, WNL	Aspiration pneumonia, secondary to diagnostic procedure
After 2	SO_x	8 days prior, WNL	Gross, WNL	Renal shutdown with septicemia

[a] Bloch *et al.* (4)
[b] Within normal limits, WNL.

Table 8. Average Hematocrit, Carboxyhemoglobin, and Methemoglobin Levels[a]

Treatment	Hematocrit (%)	SD	Carboxyhemoglobin (%)	SD	Methemoglobin (%)	SD
Clean air	45.2	4.6	1.95	0.45	0.33	0.29
R	48.1	3.8	10.56	1.37	0.36	0.42
I	45.9	4.7	9.30	1.41	0.89	0.74
SO_x	42.8	5.5	1.86	1.04	1.29	0.41
R + SO_x	47.7	3.3	10.50	1.10	0.69	0.87
I + SO_x	46.3	4.5	9.74	1.26	0.71	0.85
NO_L + NO_{2H}	42.6	3.8	1.82	0.99	0.66	0.86
NO_H + NO_{2L}	41.9	4.8	2.18	0.61	0.98	0.45

[a] Bloch *et al.* (4)

Table 9. Cardiovascular Function of Each Group 3 Years after Termination of Exposure
(______ ± 1 SD; number of dogs in group is in parentheses)[2]

	Groups							
	1 Control	2 R	3 I	4 SO_x	5 R + SO_x	6 I + SO_x	7 $NO_L + NO_{2H}$	8 $NO_H + NO_{2L}$
Heart rate	166.7 ± 5.8 (12)	174.0 ± 11.1 (10)	145.0 ± 9.3 (4)	170.6 ± 8.0 (8)	159.4 ± 9.4 (9)	140.0 ± 11.0 (11)	161.7 ± 14.7 (6)	152.5 ± 6.0 (10)
Stroke volume	9.9 ± 0.7 (12)	9.6 ± 0.5 (10)	13.0 ± 2.5 (4)	10.1 ± 0.6 (8)	10.8 ± 0.9 (9)	10.7 ± 0.8 (11)	8.3 ± 0.9 (6)	9.3 ± 0.6 (10)
Cardiac output	1.6 ± 0.06 (12)	1.6 ± 0.06 (10)	1.8 ± 0.18 (4)	1.7 ± 0.09 (8)	1.6 ± 0.06 (9)	1.5 ± 0.07 (11)	1.3 ± 0.06 (6)	1.4 ± 0.07 (10)
Cardiac index	0.31 ± 0.02 (12)	0.29 ± 0.02 (10)	0.33 ± 0.03 (4)	0.33 ± 0.03 (8)	0.28 ± 0.01 (9)	0.27 ± 0.02 (11)	0.25 ± 0.02 (6)	0.26 ± 0.01 (10)

Table 10. Mean Intravascular Pressures of Each Group 3 Years after Termination of Exposure (mm Hg ± 1 SD; number of dogs in group is in parentheses)

Site[a]	Groups 1 Control	2 R	3 I	4 SO_x	5 R + SO_x	6 I + SO_x	7 $NO_L + NO_{2H}$	8 $NO_H + NO_{2L}$
$P_{ao\text{-}s}$	161.4 ± 5.9 (12)	175.4 ± 6.1 (10)	148.2 ± 7.8 (4)	165.8 ± 5.7 (8)	162.2 ± 6.9 (9)	162.7 ± 5.6 (11)	157.8 ± 5.3 (6)	156.6 ± 6.1 (10)
$P_{ao\text{-}d}$	108.5 ± 4.6 (12)	118.5 ± 3.7 (10)	101.1 ± 3.7 (4)	113.2 ± 4.1 (8)	103.8 ± 4.6 (9)	107.1 ± 3.4 (11)	103.2 ± 4.8 (6)	104. 3 ± 4.3 (10)
$P_{\overline{ao}}$	127.2 ± 4.3 (12)	139.8 ± 4.3 (10)	119.5 ± 5.0 (4)	136.0 ± 4.7 (8)	124.4 ± 5.5 (9)	127.6 ± 4.7 (11)	123.8 ± 4.9 (6)	123.6 ± 5.1 (10)
$P_{pa\text{-}s}$	27.9 ± 2.7 (12)	31.2 ± 3.3 (10)	24.5 ± 9.5 (4)	26.4 ± 2.4 (8)	27.8 ± 1.6 (9)	22.8 ± 3.4 (11)	28.7 ± 4.2 (6)	26.6 ± 3.1 (9)
$P_{pa\text{-}d}$	12.8 ± 2.0 (12)	14.9 ± 2.0 (10)	10.2 ± 5.2 (4)	13.2 ± 2.2 (8)	11.1 ± 1.2 (9)	8.7 ± 2.5 (11)	12.2 ± 3.4 (6)	12.1 ± 2.3 (9)
$P_{\overline{pa}}$	18.9 ± 2.3 (12)	20.2 ± 2.6 (10)	15.6 ± 7.3 (4)	18.6 ± 2.1 (8)	18.4 ± 1.3 (9)	14.7 ± 2.8 (11)	19.5 ± 3.8 (6)	18.3 ± 2.5 (9)
P_{wedge}	6.1 ± 2.0 (12)	2.8 ± 1.0 (10)	-0.1 ± 1.5 (4)	2.0 ± 1.3 (8)	2.1 ± 0.9 (9)	2.0 ± 1.0 (11)	5.5 ± 2.5 (6)	3.5 ± 1.7 (9)
$P_{rv\text{-}s}$	29.6 ± 2.9 (12)	31.6 ± 2.7 (10)	25.9 ± 9.6 (4)	28.0 ± 2.2 (8)	31.0 ± 1.8 (9)	24.5 ± 2.6 (11)	34.8 ± 4.1 (6)	29.0 ± 3.3 (10)
$P_{rv\text{-}d}$	-1.22 ± 0.53 (12)	0.04 ± 0.43 (10)	-2.93 ± 0.54 (4)	-2.77 ± 0.36 (8)	-0.35 ± 0.49 (9)	-1.53 ± 0.52 (11)	-2.28 ± 0.69 (6)	-0.48 ± 0.95 (10)
$P_{\overline{rv}}$	10.5 ± 1.3 (12)	10.5 ± 1.3 (10)	7.7 ± 4.4 (4)	9.2 ± 1.2 (8)	10.8 ± 1.2 (9)	1.5 ± 1.4 (11)	11.4 ± 2.4 (6)	9.2 ± 1.7 (10)

[a] Site of pressure measurement: aortic systolic ($P_{ao\text{-}s}$), aortic diastolic ($P_{ao\text{-}d}$), aortic mean ($P_{\overline{ao}}$), pulmonary artery systolic ($P_{pa\text{-}s}$), pulmonary artery diastolic ($P_{pa\text{-}d}$), pulmonary artery mean (P_{pa}), pulmonary artery wedge (P_{wedge}), right ventricular systolic ($P_{rv\text{-}s}$), right ventricular diastolic ($P_{rv\text{-}d}$), right ventricular mean ($P_{\overline{rv}}$).

Table 11. Mean Values and Standard Errors of Peripheral Vascular Resistance (R_{per}), Pulmonary Vascular Resistance (R_{PV}) and Left Ventricular Work (W_{LV}) for Each Group 3 Years after Termination of Exposure (resistance in dynes-sec/cm^{-5} and work in 1 mm Hg/min; number of dogs in group is in parentheses)

	Groups							
	1 Control	2 R	3 I	4 SO_x	5 R + SO_x	6 I + SO_x	7 $NO_L + NO_{2H}$	8 $NO_H + NO_{2L}$
R_{per} ($\times 10^{-1}$)	650.7 ± 44.7 (12)	693.3 ± 25.4 (10)	571.8 ± 75.9 (4)	658.4 ± 62.3 (8)	605.2 ± 45.2 (9)	734.8 ± 60.0 (11)	787.2 ± 76.2 (6)	724.1 ± 53.2 (10)
R_{PV}	641.3 ± 52.6 (12)	856.6 ± 90.4 (10)	749.5 ± 45.8 (4)	782.9 ± 122.9 (8)	787.8 ± 71.6 (9)	695.3 ± 124.3 (11)	944.8 ± 225.8 (6)	801.4 ± 62.9 (9)
W_{LV}	206.1 ± 13.1 (12)	230.4 ± 16.3 (10)	219.8 ± 33.3 (4)	233.2 ± 16.7 (8)	209.5 ± 15.5 (9)	185.2 ± 12.4 (11)	159.0 ± 8.4 (6)	177.5 ± 17.5 (10)

difference in any of the groups' values except for left ventricular work. Beagles exposed to nitrogen oxides had lower cardiac work values than did those exposed to SO_x and raw auto exhaust.

Pulmonary function, Cincinnati: Investigators in Cincinnati had previously measured the pulmonary function of these beagles after 18, 36 and 61 months of a 16-hour per day, 7-day per week exposure regimen. After 18 months, Vaughn and co-workers (5) found no differences in $D_{L_{CO}}$, $C_{L_{dyn}}$, or total pulmonary resistance in these beagles. After 36 months, Lewis and co-workers (6) measured a lower $D_{L_{CO}}$/TLC (total lung capacity) ratio in the groups that breathed NO_L + NO_{2H} than in control beagles, and after 61 months (near the end of exposure), they reported the following:

1. Dogs exposed to auto exhaust without addition of SO_x had higher mean $D_{L_{CO}}$/TLC and $D_{L_{CO}}$ values than those receiving mixtures of NO and NO_2 (0.009 versus 0.008 ml/min/mm Hg and 9.91 versus 8.13 ml/min/mm Hg respectively), and the NO_2-high had lower $D_{L_{CO}}$/TLC ratio than control dogs.

2. Dogs that received auto exhaust had greater pulmonary capillary blood volumes than controls (19.8 and 17.2 ml, respectively).

3. Dogs receiving R + SO_x, R, and NO_H + NO_{2L} had larger mean RV values (280, 267 and 258 ml, respectively) than those receiving I + SO_x, NO_L + NO_{2H}, control I, and SO_x (239, 235, 232, 220 and 212 ml, respectively).

4. Dogs that breathed I had a larger mean nitrogen washout than control dogs (1.77% and 1.44% respectively), and the SO_x values were lower than the control group (1.14% and 1.44%, respectively).

5. Dogs that breathed I or I + SO_x had a greater R_{pul} (expiratory) than control and SO_x groups.

6. A low peak flow rate was reported for the NO_2-high group when compared to controls.

7. These authors adusted the $D_{L_{CO}}$ value to a percent of predicted, and found that none of the controls (0 to 18) were below 80% of their predicted value and that 5 of the 11 NO_2-high dogs were. Their prediction formula (19) was $D_{L_{CO}} = 0.0058\ TLC + 1.7$.

8. Six dogs that breathed R + SO_x had a larger mean RV/TLC ratio than predicted, while only one control dog had a larger ratio.

Pulmonary function, UC-Davis: There was no difference in the body weight (range of means 9.2 to 10.2 kg) (Table 12), PCV (range of means 37.3 to 40.9%), or Hb (range of means 14.2 to 15.8 g %) of the eight groups of beagles. Table 13 shows the blood gas and pulmonary function values related to ventilation during a period of spontaneous breathing soon after induction of anaesthesia. The Pa_{CO_2} of groups R, R + SO_x, I + SO_x, and NO_2-low was significantly greater than that of the control group. The same groups and SO_x had greater V_D (Table 13) and $\dot{V}_D$ than the controls. SO_x had higher respiratory frequency, and R and NO_2-low had significant acidosis.

Table 12. Body Weight (WT), Blood Gases (pHa), Breathing Frequency (f), Tidal Volume (V_T), Minute Volume ($\dot{V}_{min}$), Percent Dead Space (% V_D), Dead Space (V_D), and Dead Space Ventilation ($\dot{V}_D$) of Beagles (mean value ± 1 SE; number of dogs in group is in parentheses)

	Groups							
	1 Control	2 R	3 I	4 SO_x	5 R + SO_x	6 I + SO_x	7 NO_L + NO_{2H}	8 NO_H + NO_{2L}
WT(kg)	9.2	9.9	10.0	10.0	9.4	10.2	9.5	9.7
	± 0.4 (12)	± 0.4 (9)	± 0.6 (5)	± 0.3 (8)	± 0.5 (10)	± 0.4 (11)	± 0.4 (6)	± 0.3 (10)
Pa_{O_2} (mm Hg)	84.7	77.1	99.4	85.0	74.3	83.8	96.0	73.2
	± 5.5 (12)	± 4.9 (9)	± 9.8 (5)	± 5.6 (9)	± 4.5 (10)	± 4.5 (11)	± 11.1 (6)	± 5.8 (10)
Pa_{CO_2} (mm Hg)	37.9	46.8	40.7	41.8	47.0	45.3	41.2	44.8
	± 2.0 (12)	± 1.4 (9)	± 2.6 (5)	± 2.1 (8)	± 2.0 (10)	± 1.8 (11)	± 2.5 (6)	± 1.4 (10)
pHa	7.371	7.322	7.364	7.303	7.333	7.364	7.348	7.325
	± 0.018 (12)	± 0.009 (9)	± 0.016 (5)	± 0.010 (8)	± 0.013 (10)	± 0.017 (11)	± 0.023 (6)	± 0.010 (10)
f(breaths/min)	21.9	16.6	20.2	23.7	19.6	17.2	27.0	18.5
	± 3.9 (12)	± 1.6 (9)	± 2.8 (5)	± 3.5 (7)	± 2.5 (10)	± 1.2 (11)	± 5.0 (6)	± 2.9 (10)
V_T (ml)	78.5	108.3	101.1	111.1	98.2	102.2	88.6	93.9
	± 5.8 (12)	± 9.5 (9)	± 7 .4 (5)	± 8.0 (8)	± 8.9 (10)	± 5.8 (11)	± 6.8 (6)	± 4.4 (10)
$\dot{V}_{min}$ (ml/min)	1662	1708	1970	1039	1768	1749	2244	1787
	± 300 (12)	± 127 (9)	± 146 (5)	± 393 (7)	± 158 (10)	± 152 (11)	± 339 (6)	± 250 (10)
% V_D	58.4	67.0	57.3	63.0	68.4	63.7	64.8	66.2
	± 2.6 (12)	± 1.6 (9)	± 4.4 (5)	± 2.5 (8)	± 1.6 (10)	± 2.6 (11)	± 3.3 (6)	± 2.3 (10)
V_D (ml)	45.1	72.0	57.5	69.6	66.6	62.4	56.8	65.3
	± 3.1 (12)	± 6.4 (9)	± 5.2 (5)	± 5.0 (8)	± 5.4 (10)	± 3.3 (11)	± 3.5 (6)	± 3.1 (10)
$\dot{V}_D$ (ml/min)	954	1146	1135	1588	1221	1104	1319	1198
	± 168 (12)	± 105 (9)	± 143 (5)	± 257 (7)	± 129 (10)	± 95 (11)	± 264 (6)	± 187 (10)

Table 13. Total Dead Space of Control Group Compared with Exposed Groups 2 Years after Termination of Exposure

	Total dead space (ml)
Control	45.1
R	72.0
SO_x	69.6
R + SO_x	66.6
NO_H + NO_{2L}	65.3
I + SO_x	62.4

Figure 1 shows R_{rs}, R_{pul}, and R_{cw} of the beagles. The R + SO_x group had higher R_{rs}, R_{pul}, and R_{cw} values than any of the other groups whose values were similar.

The $D_{L_{CO}}$ of all the groups was similar, but when normalized with TLC, the ratio $D_{L_{CO}}$/TLC was smaller in all groups exposed to air pollutants than that of the control group (Table 14). The mean pulmonary capillary blood volume (V_C) was 15.6 ± 2.0 ml in the control group and 17.1 ± 4.3 ml in the exposed groups. There was no difference between any of the groups' V_C values.

Table 15 shows the mean values of inspiratory and expiratory quasistatic lung and chest wall compliances (C_{L_I}, C_{L_E}, andC_{cw_E}). The C_{cw_E} was similar in the control and R + SO_x groups, but in all other exposed groups, C_{cw_E} was less than that of the control group.

Figure 2 shows the mean values for total lung capacity (TLC) and the lung volume subdivisions (VC, FRC, and RV) for each group. The mean TLC was similar in all the exposed groups, but the SO_x, R and NO_2-high groups had significantly larger TLC than that of the control group. There was no difference in the FRC measured by the N_2-equilibration or the Boyle's law technique, and there was no difference between the FRC of the various groups. Inspiratory capacity (IC) was greater than that of the controls in the SO_x and R groups. The RV was similar in all exposed groups, and was greater than that of the controls except I + SO_x.

Figure 3 shows the relationship between dynamic compliance (C_{dyn}) and frequency (f) for each group. There was no difference in the slope C_{dyn}/f for the control, I + SO_x, R + SO_x, I and R groups, but the SO_x, NO_L + NO_{2H} and NO_{2L} groups had greater changes in C_{dyn} with increasing f than did the controls. The differences appear to be due to the high C_{dyn} at low frequencies in the SO_x, NO_L + NO_{2H} and NO_{2L}.

Tables 16, 17 and 18 show the pulmonary function values for the control beagles in this study compared to those from a group of healthy beagles previously studied in this laboratory. Most values are very similar.

Table 19 shows the mean pulmonary function values for each group done immediately after termination of exposure (TE) (6) and our values (2YR) done 2 years after termination of exposure. We have included all comparable values for each group for the two test periods, although techniques were not always identical. Values for a group that appeared to have changed between TE and 2YR are underlined in Table 19. We compared each dog's TE lung

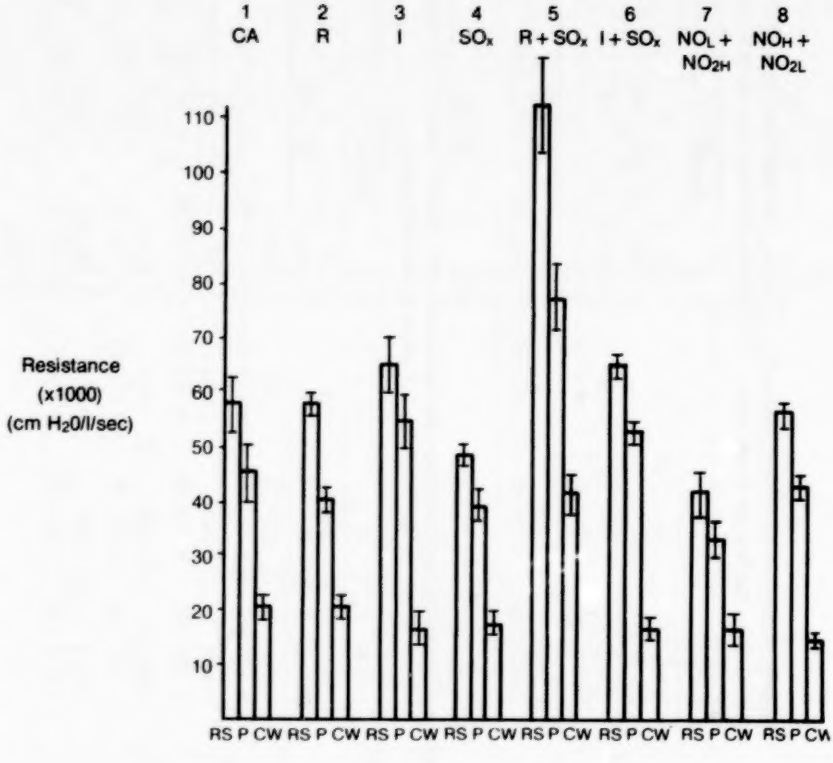


Figure 1
Mean values with standard errors for respiratory (rs), pulmonary (p), and chest wall (cw) resistances for each group of beagles.

Table 14. Pulmonary Diffusion Capacity (D_{LCO}) and D_{LCO} to Total Lung Capacity (TLC) Ratio (D_{LCO}/TLC) for the Beagles (mean value ± 1 SE; number of dogs in group is in parentheses)

	Groups							
	1 Control	2 R	3 I	4 SO_x	5 R + SO_x	6 I + SO_x	7 NO_L + NO_{2H}	8 NO_H + NO_{2L}
D_{LCO} (ml/mm Hg/min)	12.3 ± 0.6 (12)	12.4 ± 0.7 (8)	10.5 ± 0.7 (5)	13.3 ± 1.0 (8)	11.8 ± 0.6 (10)	11.2 ± 0.7 (11)	9.8 ± 0.6 (6)	11.3 ± 0.7 (10)
D_{LCO}/TLC (X1000)	11.3 ± 0.7 (12)	9.1 ± 0.6 (8)	8.7 ± 0.9 (5)	9.1 ± 0.8 (8)	8.9 ± 0.5 (10)	8.8 ± 0.7 (11)	7.1 ± 0.5 (6)	9.0 ± 0.7 (10)

Table 15. Inspiratory and Expiratory Lung and Chest Wall Quasistatic Compliance Values for the Beagles (ml/cm H_2O ± 1 SE; number of dogs in group is in parentheses)

	Groups							
	1 Control	2 R	3 I	4 SO_x	5 R + SO_x	6 I + SO_x	7 NO_L + NO_{2H}	8 NO_H + NO_{2L}
C_{LI}	39.6 ± 4.0 (12)	44.3 ± 4.0 (9)	34.6 ± 2.0 (5)	44.2 ± 2.0 (8)	33.5 ± 2.0 (10)	37.2 ± 2.0 (11)	42.2 ± 4.0 (6)	38.1 ± 2.0 (10)
C_{LE}	65.7 ± 8.0 (12)	74.7 ± 8.0 (9)	88.8 ± 6.0 (5)	78.6 ± 5.0 (8)	54.2 ± 3.0 (10)	60.6 ± 4.0 (11)	74.7 ± 8.0 (6)	62.9 ± 2.0 (10)
C_{CWI}	154.9 ± 15.0 (12)	156.6 ± 20.0 (9)	141.0 ± 15.0 (5)	175.5 ± 12.0 (8)	153.7 ± 18.0 (10)	152.3 ± 13.0 (11)	127.5 ± 19.0 (6)	151.8 ± 18.0 (10)
C_{CWE}	253.0 ± 27.0 (12)	150.0 ± 19.0 (9)	144.6 ± 19.0 (5)	166.5 ± 19.0 (8)	232.5 ± 23.0 (10)	158.7 ± 12.0 (11)	128.8 ± 10.0 (6)	151.3 ± 16.0 (10)

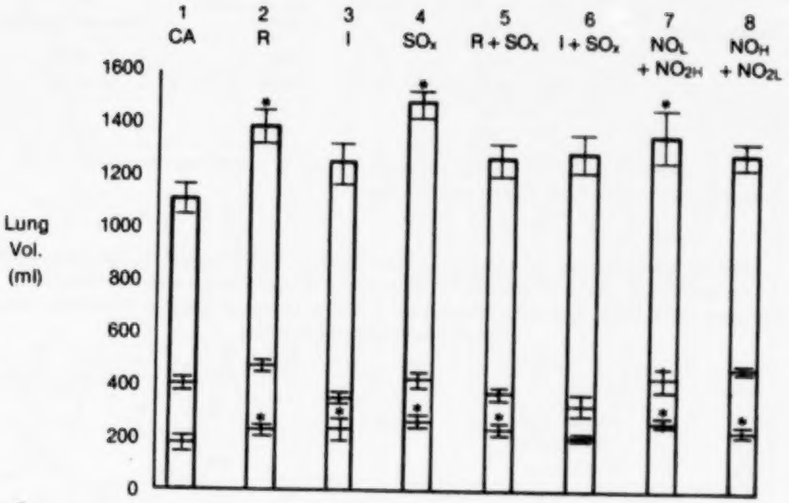


Figure 2
Mean values with standard errors for total lung capacity, functional residual volume, and residual volume (top to bottom of each bar) for each group of beagles (* = significant).

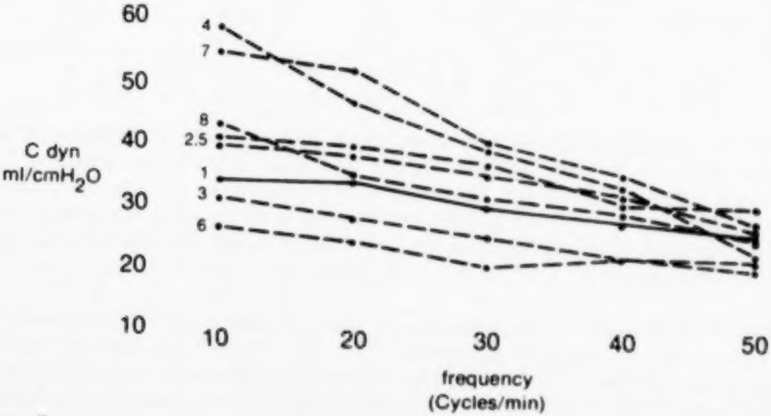


Figure 3
Mean dynamic compliance values (C_{dyn}) at different breathing frequencies for each group of beagles identified on the left of each C_{dyn}/f plot.

Table 16. Lung Volumes of the Beagles in the Control Group at the Termination of Exposure (TE) and 2 Years After Termination of Exposure (2YR) Compared with Values from a Similar Group of Beagles Previously Studied at UC-Davis (External Controls).

Volume (ml)[a]	TE	2YR	External controls
TLC	979	1095	1028
VC	819	924	932
FRC_{N_2}	297	311	373
RV	163	178	209

[a] TLC = total lung capacity; VC = vital capacity; FRC_{N_2} = functional reserve capacity measured with nitrogen; RV = residual volume.

Table 17. Compliances of Lung ($C_{LI\&E}$) and of Chest Wall (C_{CW}) of the Beagles in the Control Group at the Termination of Exposure (TE) and 2 Years after Termination of Exposure (2YR) Compared with Values from a Similar Group of Beagles Previously Studied at UC-Davis (External Controls).

Value	Units	TE	2YR	External controls
C_{LI}	ml/cm H_2O	38.8	39.0	—
C_{LE}	%TLC/cm H_2O	—	6.0	5.0
C_{CW}	ml/cm H_2O	—	249	240

Table 18. Diffusing Capacity (D_{LCO}), D_{LCO}/Total Lung Volume Ratio (D_{LCO}/TLC) and Total Pulmonary Resistance (R_{pul}) of the Beagles in the Control Group at the Termination of Exposure (TE) and 2 Years After the Termination of Exposure (2YR) Compared with Values from a Similar Group of Beagles Previously Studied at UC-Davis (External Controls).

Value	Units	TE	2YR	External controls
D_{LCO}	ml/min/mm Hg	8.6	12.3	12.2
$D_{LCO}/TLC(\times10^3)$	ml/min/mm Hg/ml	8.0	11.3	10.6
R_{pul}	cm H_2O/l/min	2.94	2.64	3.59

volumes, D_{LCO} and C_L with their 2YR values and analyzed each group's change with the Student's t-test. The RV and inspiratory C_L did not change in the control group but RV increased ($P<0.035$ to $P<0.0001$) in all exposed groups except group 3 (I) (Figures 4 and 5). TLC, VC, FRC and IC increased ($P<0.03$ to $P<0.0001$) in all groups except group 3, but increased the greatest amount in groups SO_x, I + SO_x, NO_2-high and NO_2-low. D_{LCO} and D_{LCO}/TLC increased ($P<0.03$ to $P<0.001$) in all groups except I and R + SO_x.

Table 20 shows the measured and predicted TLC values for each group. The TLC predicted TLC values were calculated with the equation TLC = 111.5 (B.W. kg), from the work of Robinson and co-workers (7). It shows that the TLC's of all groups except the control, I and I + SO_x increased 100 ml or more than predicted at 2YR. The dogs lost weight during the post-exposure period, probably due to greater activity because of less restrictive caging. The SO_x, NO_L + NO_{2H}, and R groups had a large increase in TLC despite the decrease in body weight.

Beagles Immediately Following Termination of Exposure (TE) and 2 Years After Termination (2YR).[a] (Values that tend to be different between TE and 2YR are underlined. Standard errors where available are in parentheses)

Function	Groups															
	1 Control		2 R		3 I		4 SO_x		5 R + SO_x		6 I + SO_x		7 $NO_L + NO_{2H}$		8 $NO_H + NO_{2L}$	
	TE	2YR	TE	2YR	TE	2YR	TE	2YR	TE	2YR	TE	2YR	TE	2YR	TE	2YR
C_{L_1} (ml/cm H_2O)	38.8	39.0 (0.4)	43.9	44.0 (0.4)	45.0	34.0 (0.2)	41.5	44.0 (0.2)	48.9	33.0 (0.2)	47.7	37.0 (0.2)	48.3	42.0 (0.2)	41.9	38.0 (0.2)
R_{pul} (cm H_2O/1/sec)	2.94	2.64 (0.3)	2.87	3.36 (0.1)	3.14	3.06 (0.5)	2.85	2.28 (0.2)	2.48	4.50 (0.4)	2.53	3.06 (0.2)	2.72	1.92 (0.2)	2.73	2.46 (0.1)
D_{LCO} (ml/min/mm Hg)	8.6	12.3 (0.59)	10.4	12.4 (0.73)	9.36	10.5 (0.71)	9.3	13.0 (0.99)	9.7	11.8 (0.80)	8.7	11.0 (0.68)	7.7	9.8 (0.60)	8.9	11.3 (0.67)
$D_{LCO/TLC}$ (ml/min/mm Hg/ml) (X1000)	8.0	11.0 (0.7)	9.0	9.1 (0.6)	9.0	8.0 (0.9)	8.0	9.0 (0.8)	9.0	9.0 (0.5)	7.0	8.0 (0.2)	7.0	7.0 (0.5)	8.0	9.0 (0.7)
FRC N_2 (ml)	347	339.6 (15.3)	414	412.7 (17.6)	369	396.2 (24.8)	370	439.1 (19.9)	418	401.4 (17.8)	376	384 (15.5)	402	429.0 (22.9)	408	383.7 (11.0)
ERV (ml)	142	174 (21.1)	152	167.7 (19.5)	149	181.2 (31.2)	159	175.7 (27.6)	138	162.7 (13.4)	137	185 (18.1)	167	174.0 (29.9)	149	158.3 (18.4)
IC (ml)	727	755.9 (45.2)	775	968.3 (52.5)	719	838.6 (55.2)	773	1011.0 (27.9)	734	836.6 (657.8)	790	883.5 (42.4)	782	905.1 (59.3)	767	833.8 (43.6)
RV (ml)	232	185.8 (14.0)	265	238.4 (7.2)	220	228.6 (71.6)	212	263.4 (21.4)	280	238.3 (13.2)	239	203.3 (8.6)	235	280.0 (19.4)	258	219.8 (24.1)
VC (ml)	922	930.9 (56.5)	1000	1136.1 (64)	926	1019.8 (75.6)	966	1186.6 (46.4)	943	1009.7 (67.8)	999	1068.9 (51.1)	969	1064.1 (91.8)	962	1042.1 (40.5)
TLC (ml)	1102	1095 (64.2)	1188	1374.5 (69.2)	1087	1239.8 (90.1)	1144	1450 (51.1)	1139	1248.1 (77.3)	1166	1272.2 (55.1)	1183	1342.3 (97.0)	1175	1262.5 (50.8)

[a] Lewis *et al.* (6).

[b] C_{L_1} = lung compliance; R_{pul} = total pulmonary resistance; D_{LCO} = diffusing capacity; D_{LCO}/TLC = diffusing capacity/total lung volume; FRC_{N_2} = functional reserve capacity; ERV = expiratory reserve volume; IC = inspiratory capacity; RV = residual volume; VC = vital capacity; TLC = total lung capacity.

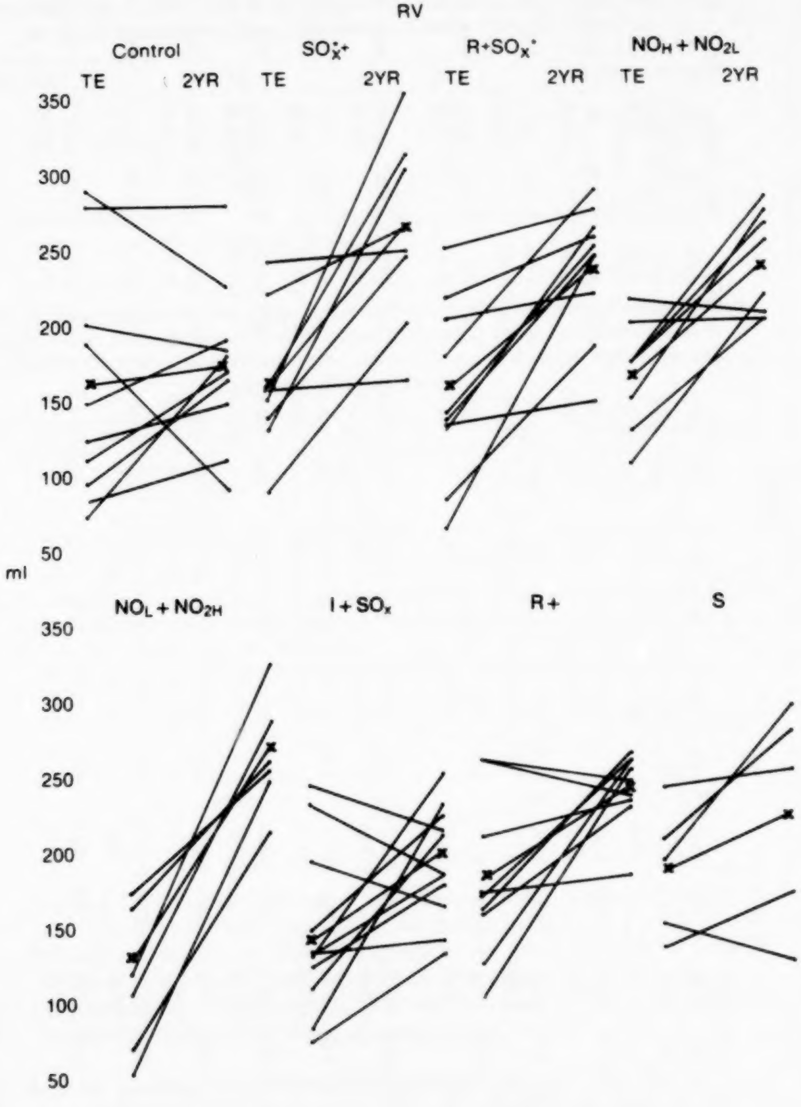


Figure 4
Changes in each dog's residual volume (RV) between termination of exposure (TE) and 2 years after termination of exposure (2YR). Bars joining x's represent mean change between TE and 2YR for each group. Groups with * are those with significant increases in RV/TLC. Those with + have significant increases in TLC.

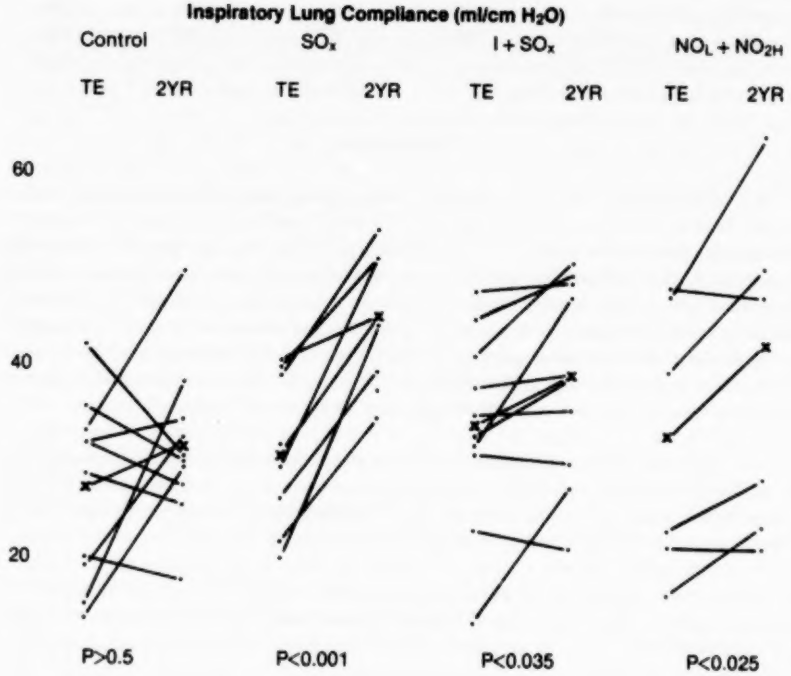


Figure 5
Changes in each dog's inspiratory lung compliance (C_L) between termination of exposure (TE) and 2 years after termination of exposure (2YR). Bars joining x's represent mean change between TE and 2YR for each group.

Table 20. Mean Total Lung Capacity (ml) of Each Group of Beagles at End of Exposure (TE, column 1), 2 Years After End of Exposure (2YR, column 2), Change in Volume Between TE and 2YR (column 3), Predicted Value from External Controls (column 4) and the Difference Between Predicted and 2YR (column 5).

Groups	1 TE	2 2YR	3 Change	4 Predicted	5 Difference
Control	979	1095	116	1028	67
R	1188	1374	186	1156	218
I	1087	1235	148	1168	67
SO_x	1144	1450	306	1168	282
$R + SO_x$	1139	1248	109	1096	152
$I + SO_x$	1166	1272	106	1191	81
$NO_L + NO_{2H}$	1183	1342	159	1108	234
$NO_H + NO_{2L}$	1175	1262	87	1132	130

Discussion

Cardiovascular function: The cardiovascular values during and following exposure did not show any clear-cut effects of the air pollutants. The small number of animals with evidence of cardiovascular dysfunction by ECG, vectorcardiography and phonocardiography; the nonspecific nature of the dysfunction; and the random distribution between groups makes the conclusion of Bloch and co-workers (4) that there was an apparent association between air pollutant exposure and positive diagnosis of cardiovascular disease equivocal. The incidence of vectorcardiographic (VCG) abnormalities (15.1%) in the exposed dogs compared to 0% incidence in controls may suggest air-pollutant effects. However, the abnormalities were not consistent nor were they seen with other functional or structural analyses.

Bloch and co-workers (4) showed that at the time of exposure to auto exhaust, the beagles had high carboxyhemoglobin and small increases in hematocrit. Lewis and co-workers (6) probably correctly associated the increased pulmonary capillary blood volume in the auto exhaust-exposed dogs with the high carboxyhemoglobin in this blood.

The cardiovascular function of all the groups of beagles studied 36 months after termination of exposure was similar and also closely related to those values of healthy beagles reported in the literature (20-23).

There appears to be no evidence of progressive cardiovascular disease in these beagles which can be unequivocally associated with the air pollutants. In retrospect, complete cardiovascular and pulmonary function measures on all beagles prior to inclusion in the study and more careful studies on each individual dog at shorter intervals might have more clearly demonstrated changes in cardiovascular function. This might have been particularly informative for those animals with demonstrable cardiovascular disease. For example, did any cardiovascular dysfunction have its onset during exposure? Did the cardiovascular dysfunction worsen during the course of the exposure? Morphological studies might have confirmed the functional evidence of myocardial lesions in individual, exposed dogs.

Pulmonary function: These studies support the hypotheses that: 1) exposure to low levels of specific air pollutants produces pulmonary injury and loss of pulmonary function, and 2) the functional loss continues following termination of the exposure. We evaluated our results in these ways: 1) comparison of internal control values to those of healthy beagles (external controls) of similar size and age, 2) comparison of each group's values 2 years after termination of exposure to their values immediately after termination, 3) comparison of each group's values to all others, and 4) comparison of each individual's values 2 years after termination of exposure to their values immediately after termination.

Comments and conclusions on group 1: The control (group 1) pulmonary function values appeared to remain nearly the same during the 2-year post-exposure period and were similar to those of other healthy beagles previously studied in our laboratory (7, 8, 9). There is evidence that many pulmonary function values of dogs relate in a predictable way to body weight (7, 24, 25) and/or within breeds to age (8, 9). There were no differences in breed, body weight, or age among the eight groups of dogs, and we concluded that these factors were not responsible for the differences in pulmonary function measured between control and exposed groups. There were no consistent or widespread pathological lesions in the lungs of the control group (26, 27). We concluded that group 1 was an acceptable representation of the healthy beagle population, and was a satisfactory control for study of the effects of air pollutants on our seven exposure groups.

General comments on exposed groups: All groups exposed to air pollutants (groups 2-8) had differences between their 2-year post-exposure pulmonary function values and their values immediately after exposure, and many of those of the controls (group 1). We did not find a clear pattern of pulmonary functional loss in the exposed groups (#2-8), although some trends were evident. In general, automobile exhaust (R or I) exposure appeared to injure airways and parenchyma, whereas SO_x, NO, and NO_2 caused injury to the parenchyma. Combinations of auto exhaust and SO_x did not appear to augment specific functional losses caused by each single species of pollutants. At 2YR there were only five dogs in group 3 (I) and this sample size was unfortunately too small for meaningful comparison to other groups.

The functional abnormalities correlate well in most instances with structural changes in the exposed groups (#2-8) (26, 27). The groups exposed to raw automobile exhaust, R (group 2) and R + SO_x (group 5), showed substantial atypical epithelial hyperplasia in their small airways. Although less extensive, these same lesions were seen in the other exposed groups. The groups exposed to higher concentrations of NO_2 (group 7) and SO_x (group 4) had the greatest airspace size and parenchymal damage as shown by several quantitative morphometric measures. The effects of the air pollutants upon the lungs of the exposed groups are the likely cause of the differences in pulmonary function values between exposed and control groups.

Because of the uncertainty of the effects of anaesthesia upon the ventilation of our beagles, it is difficult to assess the differences measured in Pa_{CO_2}, pH of arterial blood (pHa), and V_D. All the beagles were given about the same dosage of anaesthesia, and all were in light surgical anaesthesia as judged by their weak pedal pain response (21, 22). Since anaesthetic levels appeared comparable in all groups, and the control ventilatory values are at one extreme of those of all groups, the ventilatory differences in the exposed groups from control are probably the result of lung damage resulting from exposure to air pollutants. This view is supported by morphological changes in the exposed lungs and by pulmonary volume and mechanic values which differed in the exposed from those of controls and which probably are not affected by anaesthesia.

Comments and conclusions on beagles exposed to auto exhaust: Groups R and R + SO_x had similar pulmonary function response with abnormalities in ventilatory, resistance, blood gas-exchange, and lung volume values. Group 5 (R + SO_x) had the highest Pa_{CO_2}, $\%V_D$, R_{rs}, R_{cw}, and R_{pul} of all groups, which suggests these dogs had the greatest airway change. This group's C_{dyn}/f slope was not different from that of the controls. For technical reasons, we were unable to measure C_{dyn} at f's greater than 50 cycles/min. This may explain why we did not measure a greater change in C_{dyn} with increased f in this group.

The ventilatory values (Pa_{CO_2}, pHa, $\%V_D$, C_{dyn}/f, and V_D) of the R group were similar to those of R + SO_x group, and their R_{pul} increased from 2.87 to 3.36 cm $H_2O/l/sec$ and 2.48 to 4.50 cm $H_2O/l/sec$, respectively, during the 2-year post-exposure period while that of other groups remained unchanged. This suggests there was some progressive airway disease in these groups.

In all groups except 1 (control) and 3 (I), there was functional evidence of air-trapping. In groups 2 and 4-8 the RV's increased between TE and 2YR and in groups 4 (SO_x), 5 (R + SO_x) and 7 (NO_2-high) RV/TLC's increased in the post-exposure period. The TLC increased over 150 ml during the 2-year post-exposure period in groups R, SO_x, and NO_2-high. Most of the R + SO_x-exposed dogs had a high RV/TLC ratio at the end of the exposure period, and 6 of the 10 dogs in this group had RV/TLC values greater than mean control (0.17 ± 0.02 SD). The air-trapping in the R and R + SO_x groups probably is the result of airway damage with little or no parenchymal injury; that in the SO_x and NO_2-high is the result of parenchymal injury.

The I + SO_x group's and, to a lesser extent, I group's pulmonary function values were similar to those of R and R + SO_x. The C_{Li} increased in the 2-year post-exposure period in individuals in groups exposed to SO_x, NO_2-high and I + SO_x, probably signifying a change in the lung parenchyma of these dogs.

The $D_{L_{CO}}$ values for the control beagles at 2YR and the external control beagles were the same and the 2YR $D_{L_{CO}}$'s for all beagles except those in group I were greater than TE. This apparent increase in $D_{L_{CO}}$ between TE and 2YR is probably due to differences in technique at Cincinnati (TE) and UC-Davis (2YR). The $D_{L_{CO}}/TLC$ ratio increased between TE and 2YR in all beagles except those in groups I, SO_x and R + SO_x. Despite the probable increase in $D_{L_{CO}}$ values due to experimental differences between TE and 2YR, the increase in $D_{L_{CO}}$ in groups SO_x and R + SO_x did not keep pace with the increase in TLC, suggesting parenchymal damage and a real decrease in pulmonary diffusing capacity and/or increased mismatching of ventilation and perfusion.

Literature review on auto exhaust exposure: We know of no other comparable studies in which the structural and functional effects of auto exhaust alone or with SO_x have been studied during or following long-term exposure. Murphy and co-workers (28, 29) studies were on rodents exposed for brief periods (2 to 6 hr) at high levels of auto exhaust from which they reported increased total pulmonary resistance, increased mortality in the Antu-injected mice, and decreased activity during exposure. Other investigators have studied the effects of urban smog, a large portion of which is associated with auto exhaust. One of these studies by Reif and Cohen (3) showed significantly more radiological evidence of lung disease in urban dogs of 4 years and older than rural dogs of the same age. They concluded that the differences were probably due to air pollutants. Their findings in dogs exposed to urban air pollutants for a relatively long period lend support to our experimental results upon dogs exposed for 5 years. Swann and Balchum (31) studied the effects of Los Angeles smog upon total expiratory resistance of guinea pigs and reported an increase when the oxidant levels were high. They did not measure other respiratory functions, nor did their study address the long-term effects of exposure. Kagawa and Toyama (32) concluded from their studies on children exposed to Tokyo air pollution during a 6-month period that ozone, hydrocarbon, or SO_2 may affect upper airways, NO and the lower airways, and temperature may affect both upper and lower airways. They do not specify what they mean by "upper" or "lower" airways, nor would their studies specifically detect parenchymal damage.

Comments and conclusions on SO_x, $NO_L + NO_{2H}$, and $NO_H + NO_{2L}$ exposure: The SO_x group had higher TLC, RV, IC, V_D, and C_{dyn}/f values and a lower $D_{L_{CO}}/TLC$ value compared to those of controls. In addition, this group's lung volumes and RV/TLC increased while $D_{L_{CO}}/TLC$ remained unchanged between TE and 2YR. This group had the most exaggerated lung volume increase (TLC and RV), which correlated well with morphologic findings of enlarged parenchymal airspaces (26, 27). SO_x and $NO_L + NO_{2H}$ groups had substantial increases in their lung volumes during the post-exposure period. The effect of long-term exposure to SO_x seemed to be upon the parenchyma, although we detected only a very slight increase in C_{L_t} during the post-exposure period (41.5 to 44.0 ml/cm H_2O). The SO_x, $NO_L + NO_{2H}$ and $NO_H + NO_{2L}$ groups had the greatest C_{dyn}/f values, and in general all of their volume and ventilatory values were similar. Their resistance values were like those of the controls, and their most remarkable abnormalities were in lung volumes and $D_{L_{CO}}/TLC$ measurements.

Like the SO_x group, $NO_L + NO_{2H}$ and $NO_H + NO_{2L}$ groups showed the most remarkable functional and structural changes in the parenchyma (26, 27). Their structural abnormalities — hyperinflation, increased number and size of alveolar pores, and tissue destruction — are similar to the naturally occurring emphysematous lesion that Gillespie and Tyler reported in horses (33).

Literature review on SO_x, $NO_L + NO_{2H}$ and $NO_H + NO_{2L}$ exposure: The correlation of our results from SO_x, NO, and NO_2 exposures and those of others is not clear-cut due in part to differences in species studied; level, method and duration of exposure; and methods of evaluation. Others have not reported evidence of parenchymal damage like that in our SO_x-exposed dogs. Frank and Speizer (34) reviewed SO_2 exposure effects in dogs, guinea pigs and human subjects and reported increased nasal and total pulmonary resistance at SO_2 levels of about 25 ppm for 20 minutes in their dog experiments. Most other studies reviewed (35-39) were for short periods using SO_2 levels ($>$ 1ppm) higher than those in our study, and some showed increased total pulmonary resistance during SO_2 exposure. Alarie and co-workers (40) exposed monkeys and guinea pigs for 78 and 52 weeks respectively to a variety of combinations of SO_2, H_2SO_4, and fly ash. They reported the greatest change in pulmonary structure and function in those animals exposed to H_2SO_4 in concentrations as low as 0.1 mg/m^3. There was considerable variation in their pulmonary function values, and only the pulmonary flow resistance appeared to have changed in their monkeys as a result of H_2SO_4

exposure. Thomas and co-workers (41) exposed guinea pigs to 4 mg/m^3 H_2SO_4 aerosol for periods of up to 140 days. He used three particle sizes, 0.6 mm, 0.9 mm, and 9 mm. The largest particles affected only the upper respiratory tract. The 0.9-mm particles produced the greatest amount of damage — slight edema, desquamation of epithelium in minor bronchi, and reduced amounts of mucus along the bronchial tree.

Freeman and co-workers (42) reviewed the studies on the effects of NO_2 and described the parenchymal damage that they and others have seen in animals following exposure. Our dogs were exposed to comparatively low levels of NO and NO_2, but appear to have lung damage similar to that reported by others (42, 43, 44).

Pulmonary function studies upon our dogs immediately after the 68-month exposure period demonstrated modest pulmonary function changes. When these dogs were studied 2 years after the termination of exposure, many of their pulmonary function values showed an even greater difference from those of controls. The controls appeared to have stable pulmonary function values similar to those of other healthy beagles. Since these dogs were handled the same except for exposure and were of the same age and breed, we have concluded the differences in function and structure measured are due to the effects of the experimental exposure regimes.

We find the evidence for continued loss of function after termination of pollutant exposure in our dogs rather convincing since it was consistent in all groups exposed to pollutants and absent in our controls. If true, this finding should be taken into account in the design of air pollutant exposure experiments, since one may miss the effects of exposure if the animals are killed and studied soon after the termination of the exposure period. If changes resulting from exposure do require time to develop, and for that reason have been missed by investigators, this may explain in part the differences reported in the epidemiological studies of natural air pollution disasters and those from experimental exposure.

References

1. Hinners, R.G., J.K. Burkart, and G.L. Contner. 1966. Animal exposure chambers in air pollution studies. Arch. Environ. Health 13: 209.
2. Barkley, N.P., K.A. Busch, W.L. Grider, and M. Malanchuk. 1972. The concentration of lead in automobile exhaust exposure chambers. Am. Indust. Hyg. Assoc. J. 33: 678.
3. Hueter, F.G., G.L. Contner, K.A. Busch, and R.G. Hinners. 1977. Biological effects of atmospheres contaminated by auto exhaust. Arch. Environ. Health 12: 553.
4. Bloch, W.N. Jr., T.R. Lewis, K.A. Busch, J.G. Orthoefer, and J.F. Stara. 1972. Cardiovascular status of female beagles exposed to air pollutants. Arch. Environ. Health 24: 342.
5. Vaughn, T.R., L.F. Jennelle, and T.R. Lewis. 1969. Long-term exposure to low levels of air pollutants: Effects of pulmonary function in the beagle. Arch. Environ. Health 19: 45.
6. Lewis, T.R., W.J. Moorman, Y. Yang, and J.F. Stara. 1974. Long-term exposure to auto exhaust and other pollutant mixtures: Effects on pulmonary function in the beagle. Arch. Environ. Health 29: 102.
7. Robinson, N.E., J.R. Gillespie, J.D. Berry, and A. Simpson. 1972. Lung compliance, lung volumes, and single-breath diffusing capacity in dogs. J. Appl. Physiol. 33: 808.
8. Robinson, N.E., and J.R. Gillespie. 1973. Lung volumes in aging beagle dogs. J. Appl. Physiol. 35: 317.

9. Robinson, N.E., and J.R. Gillespie. 1975. Pulmonary diffusing-capacity and capillary blood volume in aging dogs. J. Appl. Physiol. 38: 647.
10. Mead, J., and C. Collier. 1959. Relation of volume history of lungs to respiratory mechanics in anaesthetized dogs. J. Appl. Physiol. 14: 669.
11. Collier, C.R., and J. Mead. 1964. Pulmonary exchange as related to altered pulmonary mechanics in anaesthetized dogs. J. Appl. Physiol. 19: 659.
12. Comroe, J.H. Jr., R.E. Forster, A.B. Dubois, W.A. Briscoe, and E. Carlsen. 1968. The lung: clinical physiology and pulmonary function tests, 2nd ed. Year Book Medical Publishers, Chicago.
13. Mead, J., and J.L. Whittenberger. 1953. Physical properties of human lungs measured during spontaneous respiration. J. Appl. Physiol. 5: 769.
14. Karp, R.B., P.D. Graf, and J.A. Nadel. 1968. Regulation of pulmonary capillary blood volume by pulmonary arterial and left arterial pressures. Circulation Res. 22: 1.
15. Ogilvie, C.M., R.E. Forster, W.S. Blakemore, and J.W. Morton. 1957. A standardized breath holding technique for the clinical measurement of diffusing capacity of the lung for carbon monoxide. J. Clin. Invest. 36: 1.
16. Roughton, F.J.W., and R.E. Forster. 1957. Relative importance of diffusion and chemical reaction rates in determining rate of exchange of gases in the human lung, with special reference to true diffusing capacity of pulmonary membrane and volume of blood in the lung capillaries. J. Appl. Physiol. 11: 290.
17. Bloch, W.N., W.C. Crocker, T.K. Wessendarp, and T.R. Lewis. 1971. Electrocardiographic telemetry with arrhythmia recording on dogs chronically exposed to air pollutants. Toxicol. Appl. Pharm. 19: 390.
18. Bloch, W.N., S. Lassiter, J.F. Stara, and T.R. Lewis. 1973. Blood rheology of dogs chronically exposed to air pollutants. Toxicol. Appl. Pharmacol. 25: 576.
19. Giammona, S.T., and W.J. Daly. 1965. Pulmonary diffusing capacity, lung volumes and pulmonary function studies in normal children, ages 4-13. Am. J. Dis. Child 110: 144.
20. Ettinger, S.J., and P.F. Suter. 1970. Pp. 126-186 *in* Canine cardiology. W.B. Saunders Co., Philadelphia.
21. Steffey, E.P., J.R. Gillespie, J.D. Berry, E.I. Eger, and E.A. Rhode. 1975. Circulatory effects of halothane and halothane-nitrous oxide anaesthesia in the dog: Spontaneous ventilation. Am. J. Vet. Res. 36: 197.
22. Steffey, E.P., J.R. Gillespie, J.D. Berry, E.I. Eger, and E.A. Rhode. 1974. Circulatory effects of halothane and halothane-nitrous oxide anaesthesia in the dog: Controlled ventilation. Am. J. Vet. Res. 35: 1289.
23. Whipple, H.E., and P.E. Van Rege. 1965. Comparative cardiology. Ann. N.Y. Acad. Sci.: 127, 155, 322, 481.
24. Spells, K.E. 1969. Comparative studies on lung mechanics based on a survey of literature data. Resp. Physiol. 8: 37.
25. Stahl, W.R. 1967. Scaling of respiratory variables in mammals. J. Appl. Physiol. 22: 453.
26. Hyde, D., D. Hallberg, A. Wiggins, W. Tyler, D. Dungworth, and J. Orthoefer. 1975. Morphometric evaluation of lungs using automated image analysis. *In* Proceedings of 6th Conference on Environmental Toxicology, Dayton, Ohio.
27. Hyde, D., A. Wiggins, D. Dungworth, W. Tyler, and J. Orthoefer. 1976. Automated measurement of airspaces in lungs from dogs chronically exposed to air pollutants. J. Microscopy, in press.
28. Murphy, S.D., J.K. Leng, C.E. Ulrich, and H.V. Davis. 1963. Effects on animals of exposure to auto exhaust. Arch. Environ. Health 7: 66.

29. Murphy, S.D. 1964. A review of effects on animals of exposure to auto exhaust and some of its components. J. Air Poll. Control Assoc. 14: 308.
30. Reif, I.S., and D. Cohen. 1970. Retrospective radiographic analysis of pulmonary disease in rural and urban dogs. Arch. Environ. Health 20: 684.
31. Swann, H.E., and O.J. Balchum. 1966. Biological effects of urban air pollution. Arch. Environ. Health 12: 698.
32. Kagawa, J., and T. Toyama. 1975. Photochemical air pollution, Its effects on respiratory function of elementary school children. Arch. Environ. Health 30: 117.
33. Gillespie, J.R., and W.S. Tyler. 1967. Capillary and cellular changes in alveolar walls of emphysematous horse lungs. Am. Rev. Resp. Dis. 3: 484.
34. Frank, N.R., and F.E. Speizer. 1965. SO_2 effects on the respiratory system in dogs. Arch. Environ. Health 11: 624.
35. Balchum, O.J., J. Dybicki, and G.R. Meneely. 1960. Pulmonary resistance and compliance with concurrent radioactive sulfur distribution in dogs breathing $S^{35}O_2$. J. Appl. Physiol. 15: 62.
36. Yokoyama, E., and K. Ishikawa. 1962. The effects of sulfur dioxide upon mechanical properties of the lungs in dogs. Jap. J. Indust. Health 4: 22.
37. Amdur, M.O. 1957. The influence of aerosols upon the respiratory response of guinea pigs to sulfur dioxide. Am. Indust. Hyg. Assoc. Quart. 18: 149.
38. Amdur, M.O. 1959. The physiological response of guinea pigs to atmospheric pollutants. Int. J. Air Poll. 1: 170.
39. Hirsch, J.A., E.W. Swenson, and A. Wanner. 1975. Tracheal mucous transport in beagles after long-term exposure to 1 ppm sulfur dioxide. Arch. Environ. Health 30: 249.
40. Alarie, Y.C., A.A. Krumm, W.M. Busey, C.E. Ulrich, and R.J. Kantz. 1975. Long-term exposure to sulfur dioxide, sulfuric acid mist, fly ash, and their mixtures. Arch. Environ. Health 30: 254.
41. Thomas, M.D., R.H. Hendricks, F.D. Gunn, and J. Critchlow. 1958. Prolonged exposure of guinea pigs to sulfuric acid aerosol. Arch. Indust. Health 17: 70.
42. Freeman, G., S.C. Crane, R.J. Stephens, and N.J. Furiosi. 1968. Pathogenesis of the nitrogen dioxide-induced lesion in the rat lung: A review and presentation of new observations. Am. Rev. Resp. Dis. 98: 429.
43. Freeman, G., S.C. Crane, N.J. Furiosi, R.J. Stephens, M.J. Evans, and W.D. Moore. 1972. Covert reduction in ventilatory surface in rats during prolonged exposure to subacute nitrogen dioxide. Am. Rev. Resp. Dis. 106: 563.
44. Haydon, G.B., G. Greeman, and N.J. Furiosi. 1965. Covert pathogenesis of NO_2 induced emphysema in the rat. Arch. Environ. Health 11: 776.

Asilomar Conference Discussion

Gillespie: The difficulty I see in Bloch's studies, and I have some tables that will show you this, is that although there were individuals in various groups that had some suggestion of abnormality in cardiovascular function, they were not consistent. The highest number of repeats was two, in which they consistently found abnormalities, and the third test often would be back to normal on individuals that were previously abnormal. The other problem I have accepting that the cardiovascular function can be related to air pollution was that they were not uniform-type abnormalities in the five affected. One finds, for example, a variety of abnormalities in a particular group, and the highest number of individuals was four in the raw automobile exhaust and there were three that had similar function abnormalities. But again, the abnormalities were not substantiated by other tests. Earlier there was some question about whether the pulmonary artery pressure or wedge pressure was high; they were not, in the same individuals. They lacked back-up data on many of these individuals to show that they were indeed abnormal and remained abnormal while in the exposure chamber.

Stara: Obviously some of the observations by Bloch may be subjective to a degree. But in a population of beagles, particularly the inbred beagle at that age, would you say that such cardiovascular abnormalities as reported by Bloch *et al.* would occur normally?

Gillespie: The 15% which they report from adding up all beagles with abnormalities in the study is higher than you would expect to see in the beagle colony at Davis or anywhere else. However, it is difficult for me to compare his abnormal values with those of others because 1) he includes very mild abnormalities, 2) repeat tests were not consistently abnormal, and 3) confirmation of abnormalities by the same or other tests were not reported. The problem with that is most of the data that you see, for example Detweiler's data on abnormalities in dogs, identify a specific lesion and give a probability in that group, so, I'm not sure that if one added up all the cardiac abnormalities that are possible, the total percentage might approach 15%.

Gillespie: . . . Expiratory compliance matched reasonably well with our external controls, and the chest wall compliance, again, matched remarkably well with our external controls. Diffusing capacity — Dr. Lewis has already mentioned that our values are consistently above his. As I remember, you mechanically inflated the lungs with negative pressure around the dog's body. I'm not sure that I would agree that would necessarily decrease diffusing capacity; I think that would increase pulmonary capillary blood volume and diffusing capacity. But for whatever reason, and I believe it probably is technical, we have different values than Cincinnati.

Brain: Theirs were done with negative pressure and yours were done with positive pressure?

Gillespie: Yes, that's right.

Lewis: When we did it with positive pressure at that point in time we got comparable results. Positive pressure opens up airways and what-have-you that may normally be collapsed.

Brain: Given the same transpulmonary pressures, I don't think the lungs will behave very differently. I see some possible differences in the pulmonary vascular bed.

Lewis: Pressures are distributed, I think, more uniformly across the lung when you do it passively than when you do it by positive pressure because you have a head pressure driving it through when you inflate the lung.

Brain: I don't think there's much evidence for that. If you plot lung volume versus transpulmonary pressure, the curves are not very different whether they are obtained with positive or negative pressure. There *are* differences in the pulmonary circulation, and I would suspect that's what might be involved.

Gillespie: I would think it would be the opposite direction. I have expected their values to be greater than ours, based upon predicted effects of their negative inflation technique. I suspect there probably was something else in different techniques. Certainly we couldn't ask for a better matching of diffusing capacity values for our external and internal controls. And again, the ratios of diffusing capability and TLC are greater at Davis, because diffusing capacities were greater.

Gillespie: Three groups — SO_x, I + SO_x, and NO_2-high — had increased compliance following the 2-year post-exposure period. All three groups had significant parenchyma lesions.

Thurlbeck: At what proportion of TLC did you measure compliance?

Gillespie: Compliance was measured at just above FRC. So, yes, it would be more like 40% of TLC.

Brain: After coming down from TLC?

Gillespie: That's right.... We feel that the animals that were exposed to SO_x and NO_2 had the most substantial change functionally at the parenchymal level. Those exposed to automobile exhaust had evidence of damage, both in the airways and the parenchyma. And the thing that we also found remarkable, and I guess maybe I should just read my conclusion, is that the abnormalities continued to occur and progressed outside the exposure chamber. And we feel that these are pretty well substantiated in that the controls' pulmonary function values were not abnormal and every exposed group showed some evidence of continued functional loss in the pulmonary system post-exposure.

Albert: Very good. I really didn't quite get clear which exposure pattern had the most damaging effect on the lung?

Gillespie: Well, I suppose the two groups I think had the most damaging effect on the lung are the NO_2-high and the SO_x, from our data. It is unfortunate that our I group, the irradiated exhaust, ended up being so small. The next two groups in order would be R + SO_x, then SO_x, as far as damage. But all exposure groups had some functional abnormality.

Lewis: In your paper you talk about fast lung volumes. I don't see those data in the paper, nor did I see the data on the slides in your presentation. Is this not a small airway-type or airway diagnostic tool?

Gillespie: The fast lung volume that we were interested in was resistance. In other words, you have to have the equipment to measure fast changes. The slow ones are, of course, something like compliance, where you inflate and deflate slowly, and all of our lung volume measurements were made with a slow procedure, so you could use a box and spirometer for those. For measurement of rapid volume changes, we used a tuned flow box. We did not measure maximum expiratory flow, for example, or peak flows, because I frankly don't think you can measure them unless you can do a tracheostomy and put a tube in the tracer within the chest. One can't do these adequately with endotracheal tubes because one will only measure the peak flow of that endotracheal tube. It's been shown that the maximum flow you can get through a 9-mm endotracheal tube is about 1 to 2 liters per second. If you do a tracheostomy, put a copper tube in the airway, you will find that a dog of beagle size will have a peak flow of approximately 6 liters per second. So we couldn't do tests on these dogs because they had to survive other tests.

Brain: Do you have data for the entire volume-pressure curve?

Gillespie: Yes.

Brain: You haven't said anything about them. I'm wondering, is there anything to be learned from them? For example, do they tell you about changes in alveolar stability? If you compare the volumes at 25 and 5 cm of water or between 25 and 2½ are those ratios any different? Is there any suggestion that stability has been affected?

Gillespie: That's a good idea. We didn't look at that, and we certainly could. We took the residual volumes and we identified functional residual capacity positions on the curve. And we have looked at the transpulmonary pressure, which is getting close to what you want, at functional residual capacity, and there was no difference between the two.

Brain: It might also be fun to plot the deflation volume/pressure curves as a percent of the TLC in order to look critically at the shape.

Gillespie: I did that with some of the groups because, again, that's the way we reported the normal beagle data in order to get the comparisons. Again, there were no differences.

Brain: What was done to the excised lobe?

Stara: That will be reported later.

Stephens: Hindsight is always better than foresight, as everyone recognizes, but I think it's unfortunate that you didn't do the physiology on the dogs that did have the pneumonectomy involved. It might have been valuable. At SRI, Dr. Freeman did some pathology on those lobes that were excised, and I did some ultrastructural work and light microscopy as well. We did not do the pathology on the recovery animals. I think Dr. Dungworth and Dr. Hyde worked on those, both on the scanning and light microscopy levels. In reading the abstracts and parts of the papers last night, I can see we use different tools, but it will be interesting to compare the data.

Gillespie: The problem I had is that it's awfully hard to take the same amount of lung each time you do a pneumonectomy, plus a lot of the mechanical studies we wanted to do depend upon there being an intact or normal pleural space which you're not likely to have following pneumonectomy. In other words, there's almost sure to be some adhesion.

Kleinerman: You only studied the dogs once?

Gillespie: Right.

Kleinerman: Is it possible, though, since you have some comparison between your studies and the studies of Trent, that you could extrapolate back to when the first reliable changes occurred in pulmonary function, either in compliance or in $D_{L_{CO}}$, in these animals?

Gillespie: Well, the biggest change seems to have occurred between the termination and the 2-year post-exposure tests.

Lewis: The problem is we didn't have enough parameters at 18 months to make the diagnosis that we could at 36 months. It's a very difficult thing to do, if not impossible. We only have three parameters — diffusion, resistance, and compliance at 18 months — that we could compare over the whole time frame, but even then, we don't have a pre-exposure value.

Kleinerman: Well, we're not talking about pre-exposure. We are talking about the changes during the period *of* exposure. But you have those three parameters. Is there any evidence that they change when you look at the data?

Albert: You said that at 36 months, 5/12 of the NO_2-high animals had a low diffusion capacity.

Lewis: That was the one and only thing that we could demonstrate at that time.

Kleinerman: But is that consistent in subsequent findings?

Lewis: Yes, at the 36- and 61-month time periods. But it would have been nice if we had had data on TLC for instance at 18 months to see whether changes had occurred at that point, but we didn't have this TLC data. I did want to bring out something that Dr. Stara asked me to talk about, and I skipped over it in trying to wrap things up. Let us dwell a little more on the case of the oxides of sulfur and perhaps their role in the etiology of cardiovascular disease, I have to somewhat agree with Dr. Gillespie on the cardiology data. I think Dr. Bloch went to great lengths to perhaps demonstrate that there was an effect of air pollutants on the cardiovascular system. But many of the criticisms that you have expressed I have weighed heavily over the years. Yet, some of the cardiac symptomatology has been confirmed, one which was validated both by enzymes and vectorcardiology and one which was diagnosed only by electrocardiography. These occurred one in $R + SO_x$ and one in $I + SO_x$. Some years ago I did a study of sulfuric acid alone. The level we used was the federal industrial air standard of 1 mg/m^3. It didn't mimic the 40-hour work week, but it did mimic continuous exposure. Although no cardiology was done, one of the significant findings that we found was both a smaller lung size, mass, volume, etc., and also a smaller mass of the heart which was significantly different in dogs exposed to sulfuric acid mist.

Brain: Is that an absolute organ weight, or an organ/body weight ratio?

Lewis: That's an absolute organ weight. There were no differences in body weight.

Brain: So there was a decrease in organ weight?

Lewis: Right. There was an atrophy apparently of both the lung and heart. I don't know if it's a pharmacologic response to a sulfate or not. I'm not building a case that oxides of sulfur cause cardiac disease. I just say that there's evidence within my own camp to suggest it.

Albert: Did those dogs show confirmatory evidence on necropsy of having had myocardial infarction?

Lewis: I didn't necropsy them personally, our veterinary pathology staff did. Once again, I don't know whether it was observed or not. The answer to your question is that no myocardial infarctions were recorded on gross autopsy.

Orthoefer: One of his myocardial infarctions was done, it was one of the trauma dogs that had died early. Dr. Bloch said it had a myocardial infarction, and pathology never confirmed it. The animal died within hours; at that time I don't think special stains would even have shown in infarction; however, they might now.

Lewis: I'm not sure that's the same case, because in his findings the concluding remarks for both dogs are "the animals gradually recovered."

Orthoefer: One of the animals that died supposedly had a myocardial infarction.

Dungworth: None of the animals that were done at the time of the scheduled killings had evidence of myocardial scars. All dogs by 9 years of age have a triad or lesions in the heart, usually of a mild degree. These are muscular arteriosclerosis in small arteries, minimal foci of myocardial fibrosis and the so-called mucoid degeneration of A-V values. Other than minimal variation in those features, there was no specific evidence of previous infarction. But that doesn't rule it out either, because the chances of finding it are slim after a considerable length of time, particularly if it were a small infarct.

Stara: The dog in question died eventually during the night, and it was found the next morning. We couldn't do satisfactory pathology.

Orthoefer: I really don't remember that particular case.

Lewis: I will repeat the statement I made the other day, namely that we could well have missed the animals that had heart disease because they were the ones that were most vulnerable to fighting and trauma and death.

Orthoefer: Actually, if we had Dr. Bloch's specific data, it's possible to go back and check out each incident. We've got to know the exact dog.

Stara: I think we should have had Dr. Bloch here. I tried to reach him and could not. He was the one who did the work, and we permitted him to take the data with him to write the papers. It was a massive amount of raw data. At the time, it was questionable if we should copy it all.

8. REVIEW OF RADIOLOGIC STUDIES

D. Dungworth

I'm not going to spend much time talking about the radiographic studies, because we can't reach satisfactory conclusions from them. There were two radiographic studies performed about 4 months apart: the first, 18 months after the dogs had arrived in Davis, that was in late fall of 1972; and the second, 4 months later in January-February, 1973. The main value of the radiographic studies, in fact, was to point out to us that if we were to provide any decent morphologic conclusions or evaluation, we would have to provide better definition of the lesions and, even more importantly, because of overlap of abnormalities found in the control and experimental groups we realized that we would have to apply numbers to the morphologic findings to enable statistical analysis. That stimulated us to develop the morphometric aspects, which later Dal Hyde will tell you about. The problem as far as the radiographic studies are concerned is that there is a background noise even in control beagles, as there is in any aging animal. This consists mostly of an increasing interstitial pattern which results from peribronchial and perivascular fibrosis. I am not going to present illustrations because the lesions were relatively minor, and they would not show up particularly well on the screen. The radiologist took the radiographs (lateral and ventrodorsal views) from the second series of examinations and in a blind study, assigned them to one of three grades of prominence of interstitial patterns (1 + through 3 +). We felt unable to make clear-cut interpretations from these data because of the small number of dogs, the overlap among the treatment groups, and the subjectiveness of the grading. The only other finding from radiology examinations was that there was a high frequency of hip dysplasia in both control and exposed beagles.

Asilomar Conference Discussion

Question: Were these lungs inflated to TLC?

Dungworth: These were done unanaesthetized because they were small dogs and because we didn't want to jeopardize their continued life. We told the radiologists that these were extremely important animals. Because there had been some anaesthetic deaths, we advised against anaesthetizing for radiography. They were done in the standard manner, at maximum inspiration. To put the grading of pulmonary abnormality noted radiographically into perspective, I should point out that the range of density was a small one and even the 3 + was less severe than the increased density that is seen in the control aged beagle population at the University of California, Davis, that lives outdoors in a somewhat dusty environment. These beagles were indoors, not in a dusty environment. Even a 3 + is, in relative proportions, mild.

Question: Are those percentages?

Dungworth: Those are percentages within the various categories and these are the numbers of beagles in each treatment group. We didn't bother getting statistical analysis on these, because there's too much variation, there's too much operator error. If we take these at face value, which we can't, there is no correlation with the subsequent morphologic and physiologic findings. This group here, the R and the R + SO_x, as Dr. Hyde will tell you, had the most obvious bronchiolar cell proliferations with no emphysema. The NO_2-high and SO_x groups here, these, as Dr. Gillespie told you, had the most evidence of parenchymal damage physiologically and, in fact, they were the two most severely emphysematous groups. Here's a group that with respect both to emphysema and proliferations in small airways were relatively close, but look at the difference in radiologic findings.

Orthoefer: It appears that probably the most severely affected was not as severely affected as normal animals in an outside environment.

Dungworth: Well yes, with respect to interstitial fibrosis, that is. We're looking at background noise here. The radiologists weren't picking up the lesions that subsequent morphological examination proved to be important, and interstitial fibrosis was not among these.

MacEwen: Did you say that the Dr. Morgan paper on hip dysplasia was from measurements made on these particular animals, but that the dysplasia was not correlated with exposure treatment?

Dungworth: Yes, it was looked at, but there was no difference between the frequency with which it was found in control animals and in the exposed. It was also about the same percentage frequency in the control beagles at Davis which came from a different breeder and so on.

Kleinerman: I think emphysema or a destructive disease in man is a difficult disease to diagnose by radiology, by the usual chest x-rays. You really can't make a diagnosis until late in the course of the disease. But I think most radiologists who do work on the chest in humans attempt to standardize a means by which they take a film and they have extreme quality control about the usability of the Roentgenogram. Also, I think they have the added advantage of having a steady state in which they take the film at full inflation. If you had both of these things available, a quiet chest with full inflation and good quality control of the type of radiograph that you took, would you feel that you could get more information from these films?

Dungworth: The quality control is good, the quality of the actual plates is good. The problem of controlled inflation we were aware of, but we did not want to anaesthetize the animals. Because this lesion is relatively mild compared to the severe cases in man, I would surmise that even if they had been anaesthetized and at a standardized inflation volume, we would not have found anything definite. Unless perhaps it is argued that we could have got numerical data by putting these films, assuming everything was standardized, on some sort of densitometer. We talked about this, but we felt it would not be justified. It is necessary to be aware, if I may use a jargon phrase, of cost-benefit analysis. You know there are some things that are going to be much more likely to produce useful information than others, and if you can't afford to follow all avenues and don't have the time and personnel to do them all, then you go with those having the highest probability of providing useful information. And on that basis, we decided it wasn't worthwhile to put a tremendous amount of effort into the radiographic studies and probably not come up with anything. That was before we saw the lungs morphologically. I don't think in hindsight I would have changed my mind.

Albert: Was there consistency in the x-ray findings for given dogs, e.g., if you found a 3+ abnormality in the first x-ray, did you find the same in the second x-ray?

Dungworth: We didn't attempt to do it twice by blind grading. The radiographs taken 4 months apart were compared for each dog, and no significant change was noted.

Albert: The reproducibility of the observations of the abnormalities would give confidence in the results, I would think.

Tyler: Basically, we have been trying to follow radiographic changes in monkeys not exposed and it's really interesting how those radiographic changes come and go. The consistency is the interesting part or the change in the lung. We worked hard at it for about 6 months I would say. We just weren't able to identify with tissue methods what the radiologists were describing. Radiologists can make some very nice descriptions based on pictures, descriptions of those radiograms. But correlating those with the tissue in our hands has been unsuccessful in other attempts. We thought, why push that issue at this point in time with these valuable dogs because we were unsuccessful in the past.

Dungworth: Again, I think the effect was not recognized. Where I put the 3+ abnormality was still less radiographically than in some of the controlled beagle population kept outdoors. We are really talking about a fairly low spectrum of changes. It's not as though massive fibrosis or translucence isn't obvious. Had they been, then I'm sure we would have gone into it more carefully.

Thurlbeck: Are you telling us that a 3+ radiologic lesion, shown to a human radiologist, would be considered normal?

Dungworth: I'm saying we see this sort of thing in older people. That's exactly what I meant. It's impossible to come up with an absolute grading. It's a relative grading among these animals.

Nettesheim: Considering these rather minor looking physiological changes, you wouldn't expect that they could see anything in the x-rays.

Thurlbeck: It's worrisome that two of the animals have a 3+ change. That signifies to me terrible looking x-rays, an equivocal change that I understand.

Dungworth: Right, and that's what I meant when I pointed out that it's a relative grading, so when he put them into three piles he had three piles. The worst pile is a very mild lesion and is commonly seen in older dogs.

Thurlbeck: Why go through the radiology exercise anyway? But of course the reason you do it — you never know before you start what you'll find.

Question: How many piles would one have in the clean air, if he had three from each dog? How many would your radiology department have?

Dungworth: Depends on what you mean by clean air. If they are in a dusty environment, where we get the same amount of dust picked up by a summer wind, in a 9- to 12-year- old population there would be 15 to 20%. None of them would be absolutely normal, relative to 6-month-old, perfectly healthy, out-of-the-colony beagles.

Question: But how many categories does your radiology differentiate?

Hueter: 0 to 3, 0 to 10, 0 to 4.

Dungworth: No. Remember this was an arbitrary scale that he devised to look at these particular 86 beagles. If you ask me if he took the same age animal from the control population at Davis, how many would have been in the 3+? I would say 20% or more.

Question: I was wondering if it was 4+, 5+ and whatever?

Dungworth: No, this didn't relate to the clinical appreciation of disease. It's purely an arbitrary thing that related to the relative abnormalities seen in this particular group of beagles. The emphasis here and throughout the study, particularly in the early phases, was looking for differences among groups.

Albert: From a practical standpoint, the issue is whether you would recommend that periodic x-rays be done in subsequent chronic studies knowing that the pulmonary function studies involve anaesthesia which has an inherent risk and that x-rays may also have risk but probably a much lower one.

Dungworth: It depends on the intensity of insult. It just so happens that in this particular study the insult was relatively low and the radiographic technique that was used was not sensitive enough to pick it up. You have to do it once at least and see about where you are on the spectrum of damage according to the level of insult.

Lewis: At least not only the level of insult but the material to which a subject is exposed — let's say if I was doing dust exposures, e.g., coal dust — I would like to have radiographic examinations.

Thurlbeck: X-rays are not worth doing for the detection of early small airways disease or minor degrees of emphysema. If on the other hand, part of the results that may be seen might be due to intermittent pneumonic episodes.... Let us speculate that these dogs got bits of pneumonia or interstitial disease of various sorts that came and went. Perhaps the answer is to do the radiology more frequently. I'm not sure how difficult this is but perhaps one should do 3-monthly x-rays as part of the physical examination of the dogs to let you know what is going on from time to time in the chamber.

Dungworth: If you have a radiology unit with everything on hand I would agree.

Orthoefer: I think there have been radiographic studies done of urban versus rural dogs. There were differences shown. This is a lot like the dogs in this study versus radiological lab dogs.

[1] *Reif, L.S. and D. Cohen. 1970. II. Retrospective radiographic analysis of pulmonary disease in rural and urban dogs. Arch. Environ. Health 12: 698.*

Gillespie: Reif and coworkers' study[1] in Philadelphia shows that downtown dogs have more lesions than country dogs.

Busch: I've had some experience with this type of data working with Dr. Lainhart when he was with NIOSH while they were studying the textile industry and exposure to cotton dust. We had x-rays on control group and cotton dust workers, but of course there is more variation in the appearance of these x-rays. They first tried a 5-point rating scale, + through + + + + + and we learned very little from it. It was a terrible test for Dr. Lainhart; furnishing him all the x-rays in blind, and having him rank them 1 to 5 acccording to their degree of damage. There were many ties but he did put them in order and the signs in this case would be rankings from 1 to 96 or however many dogs there were. We then used the rank numbers to take average ranks for each treatment group. There is a nonparametric statistical analysis which tells you whether these rank averages are statistically different. Perhaps you realize more sensitivity that way.

Albert: It occurs to me that here at Davis, with your dusty environment, you have a built-in system for evaluating long-term pulmonary clearance without any effort whatsoever. You have radiographic changes occurring in these dogs which is something that you don't see in dogs that are kept in non-dusty environments.

Dungworth: Yes, it's a very mild form of pneumoconisis but it's mild again compared to the human. We're talking about different levels of involvement.

Tyler: Dr. Orthoefer, when you looked at these lungs at autopsy, these lungs were nice pink-looking lungs that didn't look anything like city dog lungs. They're not the kind of dog lungs you see working in Philadelphia at Angel. These are very mild lesions. The other dogs we autopsy at Davis still don't look that bad. Those beagles which are outside in the dusty area just don't compare with human lungs.

Orthoefer: I agree.

Kleinerman: I think you should remember that this is the era of computerized axiotomography. If you want to find lesions by x-ray you're going to be able to find them with the available equipment. The question is, how much do you want to spend to find the lesion. The lesion can be detected even though it may be small, if you get the kind of equipment that is available for human studies.

Orthoefer: In response to Dr. Kleinerman, there is a running battle in the *New England Journal* over the use of these three-dimensional x-ray systems which are very costly. They computerize the images and come up with a computer display of a three-dimensional cross section of the body.

Dungworth: Obviously, the resolution runs out at some point.

Brain: I won't say anything about the radiographic analysis; I think we've already said quite a bit about that. When considering the cardiovascular findings, I felt very much the way Dr. Gillespie did. I find it hard to take the results very seriously. I can't imagine using the data in setting standards, for example. However, there are a few intriguing bits that may deserve a closer look. I found Dr. Gillespie's summary of the problems with the cardiovascular data quite compelling. He mentioned that the abnormalities were not reproducible in given dogs and that they were very nonspecific. The same type of abnormality was not seen consistently and the changes seen were not substantiated by other tests. I would like to add a couple more reservations. Unlike the pulmonary findings, there was no progression during the post-exposure period; this is certainly one of the most compelling features of the pulmonary function tests. There also appeared to be a random distribution of changes between the groups. I am also concerned that there was no morphological basis for the cardiovascular changes. On autopsy, there apparently were no important differences. Thus, I think it is hard to make much of the effects of these pollutant conditions on the cardiovascular system.

In contrast, I find myself impressed and excited by the pulmonary function changes. I think this is going to be a study which, when published, will be quoted by review committees charged with making important decisions about pollutant levels. The levels selected were reasonable and not too far from ambient conditions. The pulmonary function studies have been done by experienced and reliable investigators. It is hard to think of many laboratories in North America where the studies could have been better done. I am also reassured that the data are rarely baffling and are usually internally consistent. For example, if you look at the data, one can compare the changes in compliance with the changes in lung values. If you draw the volume-pressure curve of the lung and also draw the volume-pressure curve of the chest wall, you can then sum these two together to get a curve describing the total respiratory system. The functional residual capacity (FRC) is where the lung and the chest wall have an equal and opposite recoil. If there are changes in lung compliance, this helps predict what changes in lung volumes one should see. For example, if the volume-pressure shifts up and to the left, the lungs become more compliant and if the chest wall remains reasonably compliant then a higher total lung capacity should result. If one examines the alterations in RV and TLC and compares them to the compliance changes, few disturbing inconsistencies are present. The measurements of compliance and lung volume fit very well together and I find that reassuring.

There are occasional unexplained findings. For example, the change in chest wall resistance is mysterious. In group number 5, the $R+SO_X$ group, the resistance of the chest wall (the amount of pressure you need to develop to get the chest wall tissue to flow) is doubled. That's hard to understand.

A very attractive aspect of these studies is the correlation between the morphological and morphometric findings. Another good aspect of this study has been the lack of major interactions with other causes of altered pulmonary function. We have heard about looking for urban-rural gradients by comparing city and country dogs. Unfortunately, the problem of frequency and type of lung infection as another contributor to changes in pulmonary function is a major problem. This is frequently true for many human studies as well. Even when you've identified groups that differ with respect to air pollution exposure, they may still be very different in the frequency of infection, occupation, smoking, or health attitudes. This study, however, seems to be relatively free of other contributing factors. It's reassuring to hear that both in Cincinnati and Davis these dogs did not have too many serious lung infections. It seems less likely that the changes reported are secondary consequences. If air pollution, for example, altered defense mechanisms, then the animals in some groups might get more frequent and serious lung infection. In turn, that could cause the pulmonary function changes. The possibility of this mechanism seems fairly remote in these studies.

I think it's impressive that there are many different parameters which are significantly different, e.g., frequency dependence of compliance, airway resistance, lung volumes and the inspiratory compliance. Many of these changes are substantial. Another major finding is the progressive nature of the disease. Clearly, moving the animals and continuing the study past the exposure period was the right thing to do. The differences in individual animals over the past 2-year period is one of the most striking aspects.

There are certainly questions that need to be asked. What's responsible for that progression? What's going on during that 2-year period? This is a major question for everyone interested in emphysema. What processes do you initiate, what's the clock that's ticking away and why does the progression of the disease often remain quite slow and then accelerate? What's responsible for that acceleration in the absence of further insult? It will be intriguing to study such animals more thoroughly by serial sacrifice or by more repeated physiological measurements in an attempt to chronicle that 2-year period and beyond. It's tempting to ask, what would have happened if the study had been continued 2 or 3 more years? What if the investigators had had a chance to study the dogs once more? Did you catch them just as they were beginning to approach the precipice and would they drop off even more rapidly? Were things deteriorating very suddenly? There are dangers in continuing too long if one reaches end stage situations. Then at autopsy, the morphological and morphometric data may be less meaningful if the animals become very sick and have more respiratory infections. Perhaps there were some virtues as well as vices in terminating the study.

Another lesson seems to be that repeated studies on the same animal can be a very valuable and important strategy. When the data are summarized by comparing the means of groups, it is possible to lose a great deal of information. This has been demonstrated in a number of epidemiological studies as well. For example, in our own department we have a number of environmental and occupational epidemiologists who are interested in the effects of various occupational factors on the lungs. They have been carrying out studies on fire-fighting and correlating the number of knock-downs (collapses because of smoke inhalation) or other indices of exposure with pulmonary function. If you take the means of groups of individuals (of firemen in various dose categories) and compare those means, you see few significant differences. On the other hand, if you take individuals and look at their loss of pulmonary function and average these, the results are more interesting. For example, measure their FEV 1.0 every year for 4 years and then look at the average loss in FEV 1.0. That turns out to be a more sensitive indicator of exposure. Repeated measurements in the same individuals or animals focusing on the rate of loss of function over long periods of time can be very valuable.

It would be important to know about the times at which function is changing most rapidly. I think we can't conclude very much about that in this study since measurements were made infrequently. But most of the loss of function is after the exposure. Is rate of loss linear or does it accelerate as the end of life approaches? It's not surprising that these later years are very important. If you consider important human diseases such as cancer, emphysema, bronchitis, arteriosclerosis, etc. — the incidence of all these diseases increases dramatically with advancing age.

We'll soon talk in length about the choice of species and some of the problems that are involved there. Certainly in the design of future studies it is essential to try to get the most results from a finite amount of money. The question will surely be discussed tomorrow about the relative merits of dogs versus rodents and other experimental animals. In retrospect, I suppose it would have been productive if some of the advantages of dogs had been exploited more, although some of my suggestions might be unrealistic. It is possible to train dogs so that one can perform a number of pulmonary function tests repeatedly on unanaesthetized animals. If that has been done, the measurements of frequency, tidal volume, arterial P_{O_2} and P_{CO_2} might be more meaningful since we would worry less about how anaesthesia had affected the control of ventilation. Also the risk of anaesthesia would be avoided and it would become practical to make frequent repeated measurements. However, to train 80 dogs is perhaps unrealistic; the investigators who make these kinds of measurements have usually done it on fewer numbers of animals.

Lewis: You have to do it from midnight to eight in the morning too.

Brain: That's another problem. I would also like to ask whether the chronic exposures influenced the vulnerability of the animals to acute exposure to the pollutants. To what extent were there alterations in sensitivity? Were there individual animals which became more sensitive? It may be useful to focus on the most damaged animals. Is there any evidence that some dogs were unusually sensitive? On the other hand, there is also the possibility that there were adaptive changes and that some animals became less sensitive. This is certainly possible for oxidant pollutants. Also the on-and-off transience of exposure might have been an interesting area to examine in terms of pulmonary function.

Dr. Lewis listed pulmonary function tests which measure either the properties of the airways or the parenchyma. I think it may be misleading to divide them too rigidly into those two separate categories. It's very hard to think of tests which measure purely the properties of airways or purely the properties of parenchyma. There are always both primary and secondary influences. For example, you think of tests that are sometimes thought of as measuring airway properties such as airway resistance or the maximal breathing capacity (MBC). Consider a situation where the initial insult is exclusively to the parenchyma. In "pure emphysema," the emphysematous lesion characterized by loss of elastic recoil and destruction of alveolar walls diminishes support for airways. Airways will then be more collapsable and may have reduced diameters. There will then be a secondary effect on the airway resistance and the maximum breathing capacity; the peak flow during forced expiration and the entire flow volume curve may also be altered. This is not because of a direct effect on airways but because of an indirect effect on parenchyma and the support they offer airways. Similarly, if we think of tests that we ordinarily think of as measuring the properties of tissue such as compliance or diffusing capacity, indirect effects may again be important. If one has airway destruction or obstruction to the extent that a piece of lung cannot be ventilated, then the remainder of the lung will appear to be stiffer and the diffusing capacity will be reduced. It isn't because the parenchyma has been changed but simply because the parenchyma is no longer adequately served by patent airways. There are few pulmonary function tests which are specific. On the other hand, perhaps it's that very nonspecificity which makes them more sensitive. The FEV 1.0 reflects changes in both airways and parenchyma. Thus, what happens to the lung, either directly or indirectly, may alter the FEV 1.0.

Lewis: I couldn't agree with you more but I'm forced into it by the conclusion that Dr. Gillespie drew, saying these pollutants affected parenchyma and affected airways. I had to go back in my data and examine both mine and his and couldn't come up with a differentiation.

Brain: The other advantage that we have here in sorting out the site of damage in this study is the chance for morphological and morphometrical correlates. I think that's going to strengthen the conclusions that you both have about where the damage is. I'm talking more about the respiratory system viewed as a black box without any other knowledge of morphological changes. I don't necessarily disagree with your specific conclusion but I just wanted to raise indirect and direct effects as a general problem.

Lee: The transient cardiovascular effects observed by Dr. Bloch may be explained, at least partially, by our recent observations relative to hypertrophy in rats exposed to carbon monoxide at concentrations of 50 and 150 ppm for only 21 days.

Comment: I find that the notion that exposure to SO_x only produces a parenchymal damage is a little hard to understand. The SO_x exposure was a mixture of SO_2 and sulfuric acid at a mass median of 0.6 micron. SO_2 is very soluble and deposits in the upper respiratory tract. Sulfuric acid particles at 0.6 micron would grow by a factor of 2 or 3 in diameter as they entered the lung and I would expect most of those to play out on the bronchi too. Yet, the animals that showed the airway damage were exposed to exhaust, whereas the ones that showed the parenchymal-only damage were the ones that were exposed to the SO_2, at least from the physiological measurements. I was also surprised that damage from the irradiated exhaust coupled with SO_x wasn't worse than that with the raw because the irradiated exhaust has a lot of oxidant in it. I thought that would have produced a lot more damage than it apparently has.

Dungworth: There is a correlation between the morphologic and physiologic findings in that the damage caused by SO_x was at the parenchymal level.

Albert: Aren't you surprised?

Dungworth: Relative to what has been said in the literature, maybe. But I don't doubt the fact. My trouble may be interpreting it.

Albert: I think the interpretation is obvious, namely that the SO_x damaged the breathing mechanisms and the dust in the air at Davis polished these animals off.

Brain: Isn't part of the answer the reversibility of the lesions? Your scenario makes sense in terms of the immediate short-term effects. Although there may be much more upper airway damage, still, to a certain extent, damage here may be reversible. On the other hand, parenchymal damage seems to be less reversible and even progressive. The fact that a few years have passed since the end of the exposure means that the upper airway lesions may have had a chance to disappear while the parenchymal lesions may have had a chance to progress. Isn't that part of the problem and thus, part of the answer?

Albert: It could be. I'm not doubting the observations.

Brain: Perhaps upper airway changes in response to SO_2 don't persist for years.

Stara: I think that one issue should not be forgotten, namely there was approximately 0.8 micron sulfuric acid mist present in the SO_x.

Albert: That's right, but once they hit the air passages at 100% humidity and a relatively high temperature, those particles would have grown.

Brain: To what size?

Albert: By a factor of 2 or 3 in diameter.

Stara: One micron particle study was reported in guinea pigs exposed to sulfuric acid and sulfates. Some reports suggest parenchymal deposition as well.

Bhatnagar: I think I may have an answer for why the SO_x and irradiated exhaust seem to act differently. The reason for the irradiated exhaust being toxic is possibly the ozone, which generates all kinds of free radicals, superoxide specifically, which is the component of all oxidant toxicity. SO_x, specifically sulfur dioxide, is metabolized in the body or rather is chemically transformed by going through the sulfite radical HS_3 which in its own transformations, enzymatic as well as simple chemical, goes through a superoxide mechanism. If ozone or superoxide is present in the tissue and sulfur dioxide is also provided in that particular tissue, it is quite possible that sulfur dioxide transformations would compete for the superoxide free radical and reduce the concentration of the superoxide radical and thereby reduce the damage that would be caused by the superoxide radical.

Dungworth: My speculations run along the same line as Dr. Brain. I think maybe we've been misled by some of the earlier SO_2 work. You've mentioned high concentrations can corrode mucosa and cause bronchitis, whereas here we're talking about a very low level. We're looking for summation of subtle increments of damage over a period of time.

Tyler: Perhaps we haven't looked at the upper airways as carefully as we looked at the parenchyma. We looked at airways but I don't think we looked at noses.

Albert: I would have expected that with the growth of the sulfuric acid particles, a fraction of what was administered really got down to the alveoli. Probably none of the SO_2 got into the alveoli. The high potency of sulfuric acid in producing peripheral lung damage is relevant to the automobile catalyst problem because those acid particles are pretty small, in the submicron range. I think this is really the first data on chronic exposure to sulfuric acid that shows morphologic effects and functional effects.

Stara: I would like to ask for some comments on dogs' upper respiratory tract, such as the length of nares in beagles and the possible scrubbing effect of these particles in upper airways. At Davis you have done some work on this, didn't you, Dr. Dungworth?

Dungworth: We have quoted others who have done work on this with ozone but we have not investigated it ourselves.

Lewis: In our lab Dr. Vaughn did it. Bob Frank and others have studied removal of air pollutants from the nose to the alveoli. The quantity of material removed is dependent on concentration, flow rates and other variables. It's very difficult to get a good handle on it. I did want to quote from the report of the project review committee on January 1970; the three atmospheres that they recommended be discontinued at that time were ironically the SO_2 atmospheres and the two NO_x exposure atmospheres, the ones that were the most demonstrable and most consistent.

Stara: I think this may imply that the dog may scrub the larger particles better than humans and therefore you may not see as a significant an effect in the upper airway; the remaining smaller particles may deposit all the way in the parenchyma and the very small airways.

Gillespie: You have to be very careful about that because the dog is a frequent mouth breather.

Orthoefer: That's what I was going to bring up. They stay in the chambers with their mouth open a lot and somebody would have to determine how often they breathed through their nose.

Lewis: Most of the deposition for particles is dependent on the very initial point of entry where there is a point of minimal cross section and either the particles are deposited there or else they have a clean shot at getting into the lungs. That point of maximal deposition is probably before these particles have a chance to grow very much. The air is not completely saturated and the residence times are very small. They may very well grow after they reach that point. I don't think it would be surprising even if they did grow by a factor of 1, 2, or 3 that they'd penetrate and deposit in small airways as in the alveoli.

Kleinerman: You mean there is some advantage in having a longer one?

Dungworth: We did look at the surface very carefully by the scanning electron microscope and tabulate those features of damage. They were rather more prominent (not severe) in the raw rather than the SO_x. That seems to be the case; we have to change our ideas based on the past information.

Orthoefer: I do have cross sections of all the turbinates in these animals. I can't make much sense out of it as far as damage is concerned. Decalcification is somewhat damaging.

Kleinerman: Did you look for the increase in goblet cells?

Dungworth: This is being planned. Qualitatively we couldn't sort it out. The next morphometric task is to evaluate mucous apparatus.

Orthoefer: The dog is a frequent mouth breather but if you look at the diameter of the nares as opposed to the air space through the turbinate it seems to me there must be somewhat of a flow change as far as if they did breath through the nose. The nares don't seem to be nearly as big in cross section as the turbinates.

Tyler: Once you get beyond the vestibule, in reality, that common meatus is very large in a dog. It seems to me that it would be almost as big as a trachea.

Lewis: One thing you will notice is that this is a dose-response type phenomena. For example, in Dr. Coffin's infectivity studies where he used a mouse, it's much easier to demonstrate a response where you have a short airway like the mouse has from nose to alveoli than it is in the dog or an intermediate animal. You can usually find response to gaseous irritants in rodent species at a lower concentration than you can in a primate or the dog.

9. EFFECTS OF AIR POLLUTANTS ON VISUAL EVOKED BRAIN POTENTIALS IN CHRONICALLY EXPOSED BEAGLE DOGS

B.L. Johnson, J.G. Orthoefer and J.F. Stara

Abstract

The effects of raw and photochemically reacted automobile exhaust, oxides of sulfur or nitrogen, or their combinations on visual evoked brain potentials were studied in 88 chronically exposed beagle dogs. All dogs had been exposed daily for 16 hours over a 68-month period. The study groups consisted of one control group (17 dogs) exposed to pollution-free air and 7 treatment groups exposed to 1) raw auto exhaust, 2) irradiated auto exhaust, 3) raw auto exhaust plus oxides of sulfur, 4) irradiated auto exhaust plus oxides of sulfur, 5) clean air plus oxides of sulfur, 6) clean air plus oxides of nitrogen (NO_2-high), and 7) clean air plus oxides of nitrogen (NO-high). Visual evoked brain potentials were recorded from all animals using subdermal electrodes positioned in the occipital and parietal areas. All animals were immobilized with succinylcholine chloride and artificially respirated during data acquisition. Evoked brain potentials generated in response to a visual stimulus were recorded and characterized in terms of component latencies, component amplitudes, and overall amplitude. Although all seven treatment groups showed both component and overall amplitudes less than those for the control group, analysis of variance (ANOVA) indicated that the decreased amplitudes were not statistically significant ($P>0.05$) from control values. ANOVA also indicated no treatment effects on latencies of component waves of the visual evoked response.

Introduction

Epidemiological studies concerning the health effects of air pollutants have been almost exclusively concerned with the association of pollutants with pulmonary disease. Investigations concerning the effects of air pollutants on cardiac function and brain function have been few and confined to laboratory studies. Since the lungs are the initial site of entry of air pollutants into the body, it is understandable why the preponderance of air pollution health effects studies have been concerned with lung disease. Unfortunately, less attention has been given to possible consequences of air pollutants on other physiological systems; for example, the cardiovascular and nervous systems.

It is apparent from a review of the literature that air pollution studies concerned with the nervous system have been concerned almost exclusively with short-term, acute effects. Investigations concerned with the acute effects of carbon monoxide on the nervous system, and indirectly on behavior and human performance, have been reported by several investigators and have been summarized in reviews by Environmental Protection Agency (1) and the National Institute for Occupational Safety and Health (2). Of particular relevance to the investigation described in this report are the reports by Hosko (3) and Putz *et al.* (4). Hosko reported the effects of acute CO exposures on visual evoked brain potentials. Results from his study suggested that whenever the carboxyhemoglobin (COHb) level exceeded 20%, there occurred a disruption of components in the visual evoked brain potential, but not at COHb levels below 20%. Investigations by Putz *et al.* also used evoked brain potentials as an indicator of CO neurotoxicity. In this study, it was found that human auditory evoked brain potentials that were deemed "significant" by the subjects in an auditory discrimination task showed signs of disruption when COHb levels reached 5%.

A review of the literature concerning the toxicity of sulfur oxides indicates few investigations concerning the effects of SO_x on the nervous system. Indeed, the only reported studies are those of Bushtueva *et al.* (5) who found that exposure of humans to SO_2 concentrations ranging from 0.9 to 3 mg/m^3 disrupted alpha rhythms in the electroencephalogram and increased optical chronaxie at 1.5 mg/m^3. As with the CO neurotoxicity studies, the SO_x investigations concerned with possible effects on the nervous system have been short-term, acute studies.

The effects of chronic exposure to ozone on brain function were reported by Trams *et al.* (6) who utilized neurochemical indicators of neurotoxicity and Johnson *et al.* (7) who employed electroencephalographic (EEG) techniques. Both studies utilized the same beagle dogs exposed for 19 months to ozone levels ranging up to 3 ppm (8 hours daily exposure). Trams *et al.* reported a steady decline in catechol-o-methyltransferase activity with increased O_3 exposure, and Johnson *et al.* noted a decrease in EEG frequency content and a disruption of visual evoked brain potentials at O_3 exposure levels exceeding 1 ppm (16 hours daily exposure).

In summary, relatively few studies have been concerned with the possible neurotoxic consequences of long-term, chronic exposure to air pollution. The purpose of the investigation described in this report was to examine in a laboratory study the effects of air pollutants on brain function, as measured by evoked brain potentials. The reason for examining visual evoked potentials as possible indicators of brain neurotoxicity was due to 1) the use of such brain potentials as indicators of visual system disorders in human studies (8) and 2) the relative ease of data acquisition in a study involving a large number of animals.

Methods and Materials

One hundred and four beagle dogs were randomly assigned to exposure chambers which contained four dogs each as described by Hinners *et al.* (9). All dogs were from an inbred colony and were approximately 6 months old at the start of the exposure. One group of 20 dogs served as control subjects and were exposed to CBR (chemical, biological, and radiological) filtered air for the duration of the study. The remaining 84 dogs were divided equally into seven treatment groups as follows: 1) raw (non-irradiated) laboratory-produced auto exhaust (R), 2) irradiated auto exhaust (I), 3) filtered air plus oxides of sulfur (SO_x), 4) $R + SO_x$, 5) $I + SO_x$, 6) filtered air plus high nitrogen dioxide plus low nitric acid ($NO_{2H} + NO_L$), and 7) filtered air plus low nitrogen dioxide plus high nitric acid ($NO_{2L} + NO_H$). Each treatment group was exposed for 68 months, at which time the dogs were tested for signs of neurotoxicity.

The dogs were taken from their exposure chambers on a random schedule immediately prior to recording of electroencephalographic data. All dogs had been fasted 12 hours prior to testing. The testing of the dogs was conducted in a manner such that the principal investigator was blind to the treatment history of each dog. Clipping of hair on the head and weighing of each animal were performed prior to EEG recording. Hair was clipped from the occiput anteriorly to the supraorbital process and laterally to the base of the pinnae. Each animal was given Sucostrin (succinylcholine chloride) intravenously at the rate of 0.5 mg per kg of body weight. The animals were immediately intubated with a cuffed endotracheal tube and artificially respirated by a demand type mechanical respirator using a mixture of 95% O_2 and 5% CO_2. The respiration rate was constant, but was adjusted individually for each animal in order to maintain a well oxygenated state, as judged by pink tongue and mucous membranes.

Four monopolar subdermal needle electrodes were used for EEG recording from each dog. The electrodes were approximately 0.5 inch (1.27 cm) in length. Two electrodes were placed at parietal locations P_3 and P_4 (International 10-20 notation), while the other two electrodes were placed in the occipital area of O_1 and O_2. Electrodes placed in each ear flap and tied together electrically served as the reference electrode. The parietal and occipital areas were chosen for EEG recording because of the reported effects of some air pollutants (CO, O_3) on the visual system. Symmetrical electrode placements were used in order to investigate the possibility of asymmetric cortical damage. The EEGs were recorded in a quiet, well-lighted room with a Sanborn (Model 350-1500) electroencephalograph and recorded on a FM analog tape recorder (Honeywell, Model 7600). The bandwidth of the EEG amplifier-recording system was essentially flat from 0 to approximately 300 Hz.

The EEG recordings lasted 10 minutes for each dog. The first 7 minutes of data acquisition consisted of recording a background EEG during minimal sensory stimulation. The remaining 3 minutes of data acquisition consisted of recording visual evoked potentials recorded from P_3, P_4, O_1, and O_2 that were generated in response to photic stimulation from a Grass (Model PS-2) photic stimulator. The stimulator was positioned at each dog's eye level 15 inches from the animal's nose. The photic stimulator was set at minimum intensity (Intensity = 1). Sound absorbant material was placed within the photic unit in order to minimize the auditory click coincident with the gas tube discharge. The visual stimuli were presented at a constant rate of 1 flash every 2 seconds.

Averaged evoked potentials were obtained through use of the Advanced Averager program supplied with a PDP-8 computer. For each dog, the visual evoked potentials for the first 75 photic stimuli were averaged for each EEG lead by the aforementioned computer program and saved on punched paper tape for subsequent analysis.

The averaged visual evoked responses (VER) were characterized in this study by the following six measures:

1. Latency of the first negative peak (N_1)

2. Amplitude of N_1

3. Latency of the first positive peak (P_1)

4. Amplitude of P_1

5. Ratio of P_1/N_1 amplitudes

6. The root mean square (RMS) value of the VER

In order to test for treatment effects attributable to the seven groups of air pollution exposures, a univariate analysis of variance (ANOVA) (10) was used to test each of the aforementioned six parameters of the VER for statistical significance. Significant differences among treatments were to be examined following ANOVA by the use of Dunnett's test (10), which permits comparison of individual treatments with a control group.

Results

Comparison of VER data from EEG leads P_3, P_4, O_1, and O_2 showed no differences between EEG leads as concerns possible treatment effects. Therefore, to simplify the presentation of group data, only VER results from EEG lead P_4 are discussed. After 68 months of exposure the following numbers of animals comprised each treatment group: control, 17; raw auto exhaust (R), 11; irradiated auto exhaust (I), 10; oxides of sulfur (SO_x), 8; $NO_H + NO_{2L}$, 10; $NO_L + NO_{2H}$, 11; $R + SO_x$, 10; and $I + SO_x$, 11. Table 4 of Chapter 2 contains mean pollutant exposure levels for each treatment group. Figure 1 illustrates a typical averaged visual evoked potential as recorded from a control dog at EEG electrode P_4. The VER is characterized by the presence of a large positive peak, denoted by P_1, followed by a negative component N_1. Figures 2 through 6 contain treatment group means and the standard error of the mean (SE) for the latencies of N_1 and P_1, amplitudes of N_1 and P_1, and the root mean square (RMS) value of the total VER. The latter amplitude value was used as a measure of overall amplitude of the VER.

Review of Figures 2 and 3 show no treatment effects of any air pollution, singly or in combination, on the latencies of VER components N_1 and P_1. For N_1 latencies, ANOVA yielded $F(7,80) = 0.81$, $P = 0.59$, which, of course, is not statistically significant. The amplitude of P_1 was diminished in all seven treatment groups in comparison to the control group, but ANOVA yielded $F(7,80) = 1.74$, $P = 0.11$, which is again not statistically significant.

Possible treatment effects on overall VER amplitude were examined using the RMS value of the total VER. Treatment means as shown in Figure 6 are all less than the mean for the control condition, but again ANOVA showed this result to be statistically nonsignificant ($F(7,79) = 1.15$, $P = 0.34$).

In summary, although there are indications that VER amplitudes were reduced in all treatment groups when compared to the control condition, these decreased amplitudes did not reach statistical significance by ANOVA.

Discussion

The lack of effect of any air pollution treatment on brain function found in this study, as measured by evoked brain potentials, could be due to 1) lack of physiological damage to brain tissue due to treatment groups, or 2) lack of sensitivity of the VER method to detect minimal brain dysfunction. The former possibility could be true, since there is little convincing evidence in the literature to indicate neurotoxic effects for chronic air pollution levels at concentrations representative of those in this study. Since carbon monoxide and ozone were two major constituents of those treatments involving auto exhaust, the reports by Lewey and Drabkin (12), Lindenberg *et al.* (13) and Johnson *et al.* (7) are relevant to findings from this study. Lewey and Drabkin exposed six dogs to 115 mg/m^3 CO for 5.75 hours per day, 7 days

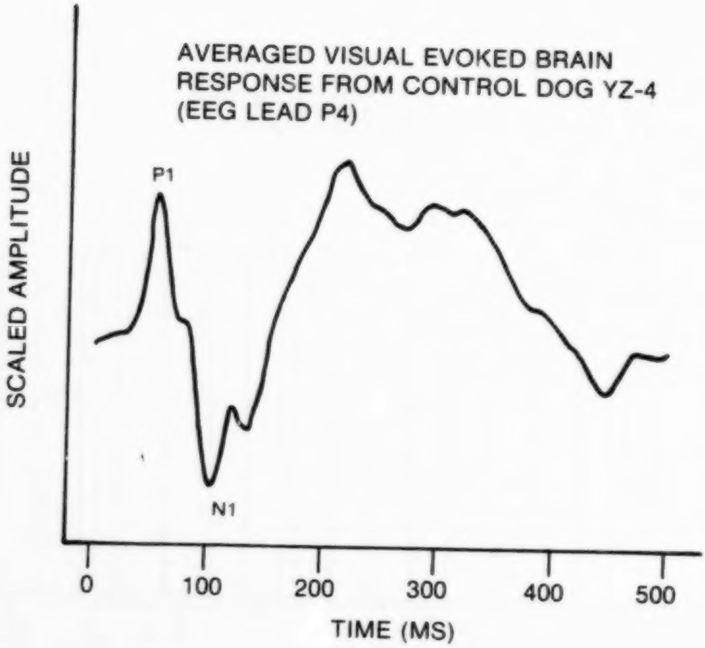


Figure 1
Typical averaged visual brain potential (VER) from a control treatment beagle dog.

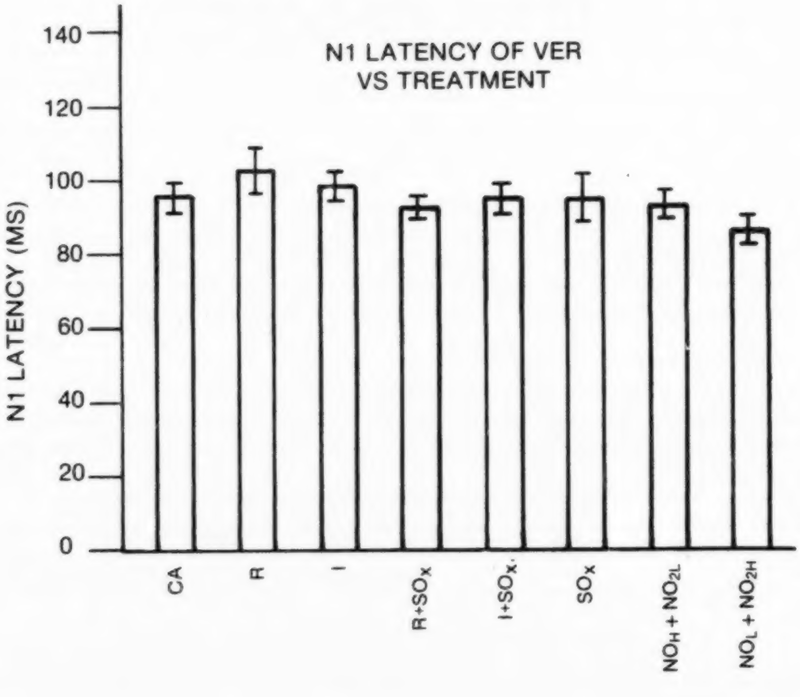


Figure 2
Means and standard errors for VER component N$_1$ latency versus air pollution treatment.

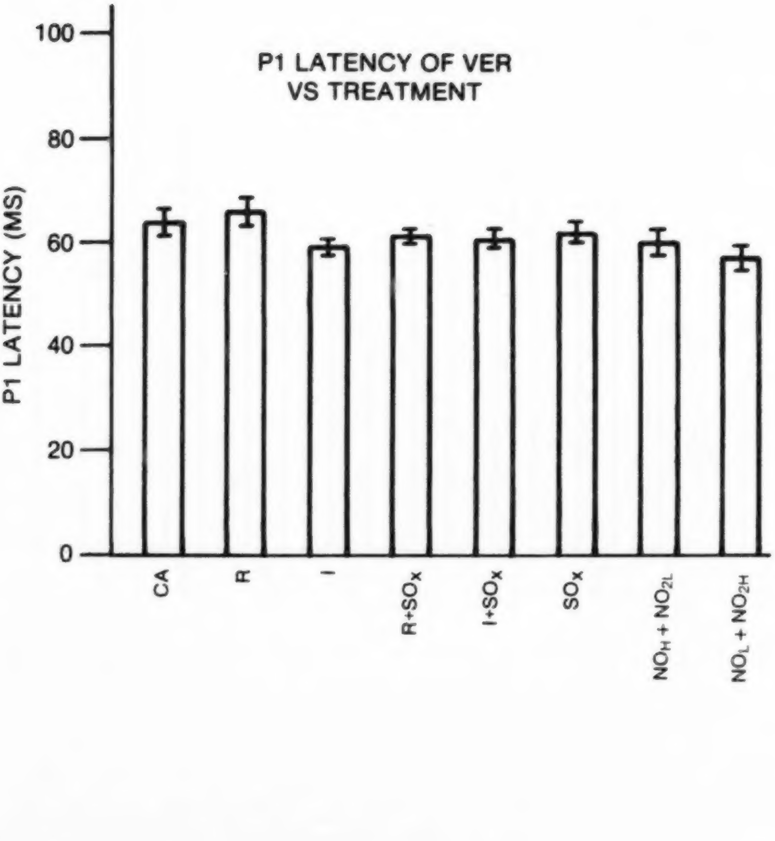


Figure 3
Means and standard errors for VER component P_1 latency versus air pollution treatment.

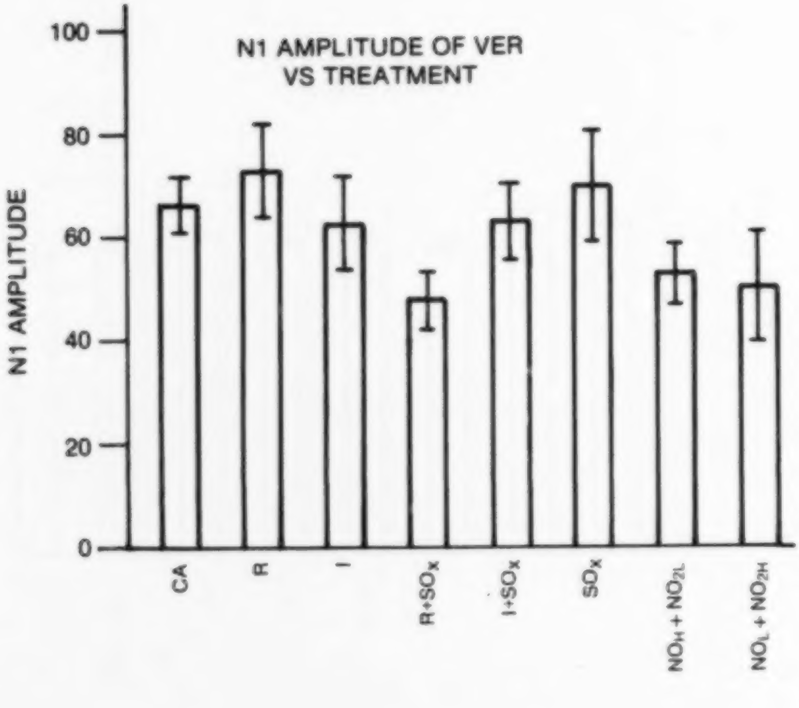


Figure 4
Means and standard errors for VER component N_1 amplitude versus air pollution treatment.

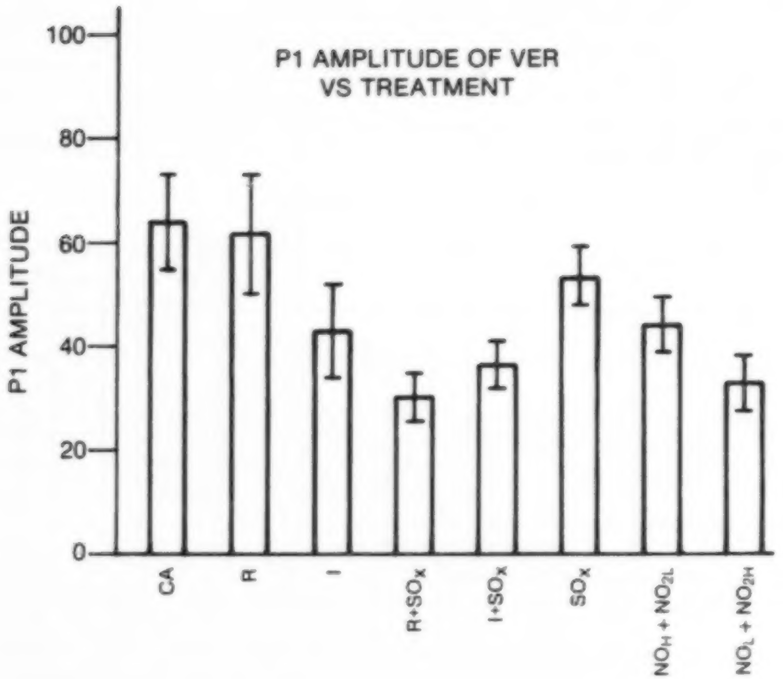


Figure 5
Means and standard errors for VER component P_1 amplitude versus air pollution treatment.

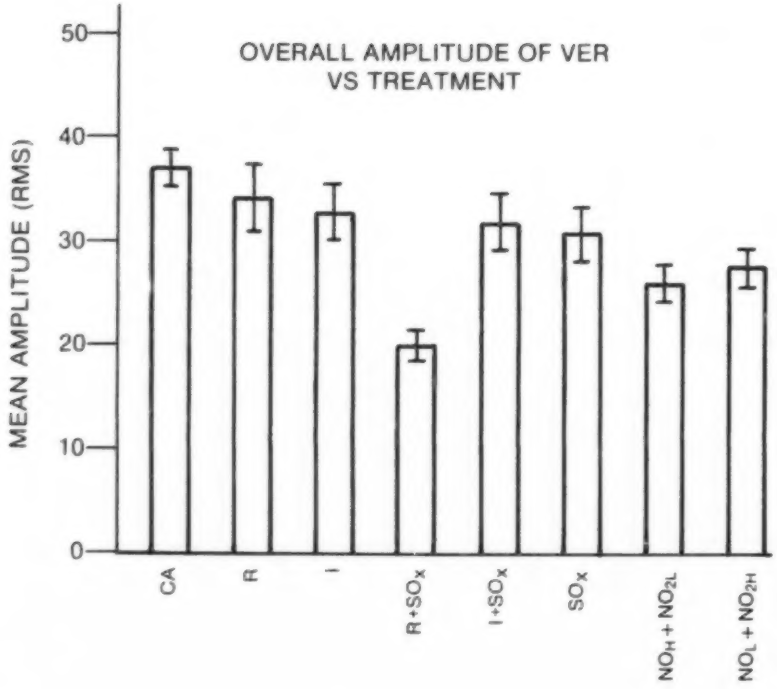


Figure 6
Means and standard errors for VER overall amplitude (RMS) versus air pollution treatment.

per week for 11 weeks and found in morphologic examination that all dogs exhibited cortical brain damage, but no EEG abnormalities. However, Lindenberg *et al.*, who exposed dogs at 115 mg/m^3 CO for 6 weeks, found no brain areas showing necrosis or demyelination. CO levels used in the study (see Table 4 of Chapter 2) were approximately equal to those of the Lewey and Drabkin and Lindenberg studies. It would therefore appear that if CO-induced damage occurs in the cerebral cortex as a consequence of chronic exposure to CO levels of 100 mg/m^3 or less, the damage will not be reflected in abnormal EEG. Johnson *et al.* found EEG changes characteristic of cortical depression in dogs exposed to O_3 for 18 months. However, the O_3 levels (2.0 mg/m^3) were considerably in excess of the ozone levels employed in the present study (0.39 mg/m^3).

As mentioned, it is also possible that minimal brain damage did occur in the dogs, but the VER method was insensitive to detecting the neurotoxic effect. Putz, Johnson and Setzer (4) found in a laboratory study of the acute effects of CO that auditory evoked brain potentials were disrupted by CO only if the auditory stimuli were "significant" to the subject. Auditory evoked brain potentials judged nonsignificant in an auditory discrimination task were unaffected by CO. These results would suggest that the sensitivity of using evoked potentials as indicators of brain neurotoxicity can be enhanced if presented to the subjects within the context of a discrimination task. This possibility, of course, was impossible to consider for use with all 88 dogs in this study, but might have been possible with a small subsample of dogs.

References

1. U.S. Department of Health, Education, and Welfare. 1970. Air Quality Criteria for Carbon Monoxide. Washington, D.C.
2. U.S. Department of Health, Education, and Welfare. 1972. Occupational Exposure to Carbon Monoxide. Washington, D.C.
3. Hosko, M.J. 1970. The effect of carbon monoxide on the visual evoked response in man. Arch. Environ. Health 21: 174-180.
4. Putz, V.R., B.L. Johnson, and J.V. Setzer. 1976. Effects of carbon monoxide on vigilance performance. National Institute for Occupational Safety and Health, Report 77-124.
5. Bushtueva, K.A. 1970. Referenced *in* Air quality criteria for sulfur oxides. U.S. Department of Health, Education, and Welfare, Washington, D.C.
6. Trams, E.G., C.J. Lauter, E.A. Brown, *et al.* 1972. Cerebral cortical metabolism after chronic exposure to ozone. Arch. Environ. Health 24: 153-159.
7. Johnson, B.L., J.G. Orthoefer, T.R. Lewis, and C. Xintaras. 1976. The effect of ozone on brain function. *In* Clinical implications of air pollution research. Publishing Sciences Group, Inc., Acton, Mass.
8. Regan, D. 1975. Evoked potentials in psychology, sensory physiology, and clinical medicine. Halsted Press, New York, N.Y.
9. Hinners, R., J.K. Burkart, and G.L. Contner. 1966. Animal exposure chambers in air pollution studies. Arch. Environ. Health 13: 609-613.
10. Winer, B.J. 1962. Statistical principles in experimental design. McGraw-Hill Book Co., New York, N.Y.
11. Lewis, T.R., W.J. Moorman, Y.Y. Yang, and J.F. Stara. 1974. Long-term exposure to auto exhaust and other pollution mixtures. Arch. Environ. Health 29: 102-107.
12. Lewey, F.H., and D.L. Drabkin. 1944. Experimental chronic carbon monoxide poisoning of dogs. Amer. J. Med. Sci. 208: 502-511.

13. Lindenberg, R. *et al.* 1962. An experimental investigation in animals of the functional and morphological changes from single and repeated exposure to carbon monoxide. Amer. Ind. Hygiene Conf., Washington, D.C., May 13-17.

Asilomar Conference Discussion

Question: What did Dr. Johnson measure?

Stara: Dr. Johnson measured the amplitude and the latency of the positive and negative peaks. Negative peak shows both latencies slightly lower than the controls and the amplitudes much lower than the controls; but using the analysis of variances, as Mr. Busch described, there were no significant differences. There were trends in all the treatment groups. This basically sums up the results.

Orthoefer: Was the Student t-test significant?

Busch: No, he didn't use it, according to what I read. He did the ANOVA test which compares all the means for excessive variability compared to the intragroup variability. He followed that up with a test called Dunnett's test which compared each treatment against the control. I don't know what results he got.

Lewis: He also did Student t-test in a random nature similar to what was done by Dr. Hyde in Davis on the morphometry and in that situation there were some values at least that were significantly different. He was reticent to make a comment on that until he felt it was mathematically sound.

10. ULTRASTRUCTURE OF BIOPSIED TISSUE

R.J. Stephens and G. Freeman

The lung biopsies to be described here at the light and electron microscope levels were obtained from female beagle dogs that were originally housed in Cincinnati and later at the University of California at Davis. Lung biopsies from 16 animals were obtained on April 26-28, 1971 in Cincinnati when exposure of the animals was terminated. Six animals were controls, five had received irradiated auto exhaust, and five had received $NO_L + NO_{2H}$. After about 30 months of recovery in clear air (at Davis, California) an additional sample was obtained from all but two of the same dogs: five controls, five that had received irradiated exhaust, and four that had been exposed to $NO_L + NO_{2H}$. These animals were sacrificed between December 12 and 19, 1973.

While these studies should not be regarded as exhaustive, they were substantial. At least five blocks were sectioned and examined at both the light and electron microscope levels for each animal from which tissue was available, both at the end of exposure and after approximately 30 months of recovery. Additional tissue was fixed in formaldehyde in Cincinnati and shipped to Dr. Freeman at Stanford Research Institute for routine paraffin embedding and pathological examination. In addition to hematoxylin and eosin, sections were also stained with aniline blue and orange G for collagen, and with acid orcein for elastic tissue.

It was not feasible to attempt to distinguish between artifactual alveolar compression and atelectasis in the tissue prepared for pathology since the biopsies, which were fixed in formaldehyde, were not filled or inflated through the airways. There were also areas of distended alveoli that may possibly have represented "compensatory emphysema." There were no appreciable differences among specimens from the three groups of dogs, whether they were designated as control, irradiated exhaust, or $NO_L + NO_{2H}$ animals.

Possible abnormalities were limited to: 1) occasional evidence of exaggerated goblet cell activation in bronchioles; 2) occasional multiple layering of bronchiolar epithelium; and 3) occasional evidence of perivascular fibrosis in all three groups. Tissue was not obtained for routine pathology at SRI after the recovery period; therefore, no data are available.

Biopsies and tissues obtained at necropsy and prepared for transmission electron microscopy were fixed in OsO_4 and embedded in Araldite. One-micron-thick sections were obtained and stained with toluidine blue for observation with the light microscope. Each block was then carefully retrimmed in specific areas of interest and sectioned for transmission electron microscopy. Because of previous experience with deep lung irritants in rats, dogs and monkeys, we concentrated our attention on the terminal airways and proximal alveolar regions. Careful examination of tissue from each animal did not reveal a significant difference among the three groups of animals, whether tissue samples were obtained immediately after exposure or after an extended recovery period. The ultrastructure of each cell type was within the limits of normal variability, and it was difficult to substantiate any consistent or significant change that could be attributed to the exposures they had received.

A comparison of data obtained from this study on beagle dogs with data from an earlier study on dogs exposed to O_3 and their controls indicated some interesting differences. In the present study, both ciliated and nonciliated cells of some terminal airways contained a number of lipid droplets. This was true regardless of whether they were controls or exposed animals. Lipid droplets were not observed in the same cells from the earlier study. However, animals exposed to O_3 at 3 ppm for 8 hours per day for 18 months showed several abnormalities. Squamous metaplasia was prominent, particularly in the respiratory bronchioles, and there was an increase in fibrosis in the peribronchiolar and periductal tissues. The cisternae of the endoplasmic reticulum in many type 2 cells were dilated and contained a proteinaceous material with a regular period of approximately 750 A°. In addition, the lamellar bodies appeared to be abnormal in cells containing the periodic material.

In conclusion, it should be noted that the total amount of tissue examined in this ultrastructural study was very small and if changes occurred that were restricted in distribution, they may have been missed.

Asilomar Conference Discussion

Stephens: . . . There's no question that there has been a very definite change in the cell type which was most prominent and recognizable in the respiratory bronchiolar area. I think, in addition, there's been a thickening of the basement laminar or basement membrane area as well. We've seen this in NO_2-exposed animals.

Albert: Is this in the dog?

Stephens: Yes, but for earlier studies with ozone.[1]

Albert: How long was the exposure?

Stephens: The exposure was 18 months and these particular micrographs were taken from animals that received 3 ppm O_3, 8 hours a day, for 18 months.

Lewis: Do you see similar lesions in the 2 x 8 or 1 x 8 or 1 x 16 in any of these treatments? That was a factorial design of 1 x 8 versus 2 x 8, etc. Did you see any lesions in lower CT (concentration x time)?

Stephens: No, we did not see this type of a change. There were some other connective tissue changes at the lower levels, but the actual epithelial changes were not seen in animals exposed to 1 and 2 ppm ozone. In the higher magnification showing the small cytoplasmic vesicles there are several characteristic desmosomes cut in tangential section but they are certainly recognizable. The tonofibrils are quite evident in the cytoplasm, clearly showing the nature of the cellular change.

---

[1] *Freeman, G.,* et al. *1973. Changes in dogs' lungs after long-term exposure to ozone: light and electron microscopy. Arch. Environ. Health 26: 209.*

Albert: Is the metaplasia present higher up in the respiratory tract?

Stephens: We didn't examine the tissue from the upper airways in these particular animals. This tissue was also acquired from Cincinnati and we did not obtain the upper airways.

Kleinerman: When you describe a lesion like this, Bob, does that mean you've seen it more than once?

Stephens: We would see this lesion on almost every slide that we would make, but it would be a small portion of the total epithelium that would be present.

Kleinerman: Your interpretation is that it's patched.

Stephens: That's absolutely right.

Albert: I don't get any feel for how much material you looked at. You remove a piece of lung; did you examine it in a systematic pattern?

Stephens: Yes, the amount of tissue obtained was substantial. It was prepared for electron microscopy. Terminal airways were embedded longitudinally and examined systematically from proximal to distal areas. Upper airways were not included.

Albert: You showed a slide that looked like a whopping increase in the number of goblet cells, then you say it really isn't increased. I just wonder whether the intensity of the examination was adequate to support the statement.

Stephens: I would have to have shown a dozen slides of each area, I suppose, to convince everyone in the room that my general appraisal was correct, but time is limited.

Nettesheim: Transmission electron microscopy is not the most convenient way of trying to determine whether there's an increase in goblet cells, though.

Stephens: I showed the high magnification light micrograph to describe the characteristics of the cell types and certainly you can find various areas along the airways that have a few more goblet cells than other areas do.

Dungworth: I think this would be more fruitful after the rest of the morphology.

11. NECROPSY

J.G. Orthoefer

Procedure

Following the final pulmonary function testing and while still under anesthesia (pentobarbital Na) the animals were exsanguinated by incising the jugular vein and brachial arteries. Immediately thereafter the lungs were removed, trimmed of extraneous tissue and weighed. The weight included all lobes of the lungs and the trachea up to the third tracheal ring.

The left diaphragmatic lobe of the lung was removed and the major bronchi were dissected out. The remainder of the parenchymal tissue from this lobe was frozen for collagen determination. The left apical lobe was separated at the major bronchi, weighed and quick frozen using liquid nitrogen.

The left primary bronchus was ligated using a heavy cotton string and the lungs were fixed by the intratracheal infusion of a cacodylate buffered glutaraldehyde-formaldehyde solution at 30 cm H_2O pressure. Figure 1 depicts the infusion apparatus. A systematic procedure was followed in dissecting the entire animal and representative samples of all tissue were saved. The weight of all organs was recorded as removed, and representative samples of some organs, and, in cases of pair organs, entire organs were frozen for later biochemical studies.

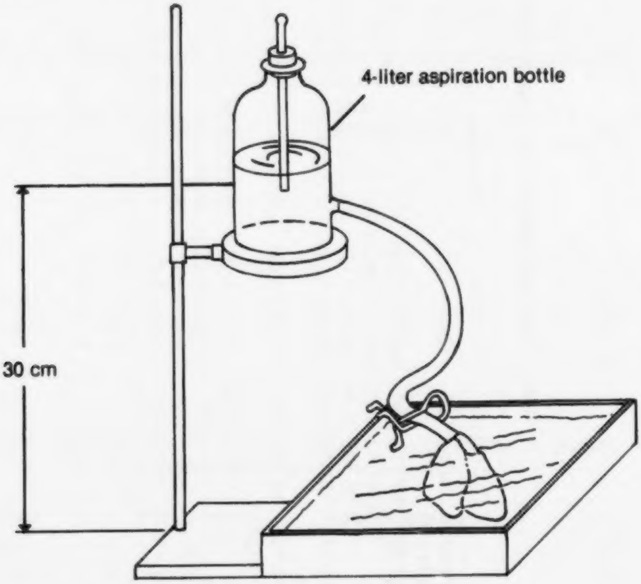


Figure 1
Fixation of the right lung was accomplished using a dilute Karnovsky's fixture at 30 cm H_2O pressure for 16 to 18 hours.

Table 1. Total Body Weights and Organ Weights and Standard Deviations for 70 Beagle Dogs

	Controls	Raw	Irradiated	SO_x	$R + SO_x$	$I + SO_x$	$NO_L + NO_{2H}$	$NO_H + NO_{2L}$
Total body weight (kg)	9.75 ± 1.80	10.90 ± 1.80	9.31 ± 2.18	10.78 ± 1.60	10.44 ± 1.78	10.62 ± 2.06	9.58 ± 1.40	9.99 ± 2.13
Lungs (gm)	102.61 ± 8.58	119.81 ± 16.85	104.90 ± 25.17	123.92 ± 12.49	114.38 ± 20.58	111.92 ± 10.85	104.53 ± 11.52	108.94 ± 12.85
Liver "	296.16 ± 63.30	365.17 ± 105.86	290.15 ± 57.58	347.24 ± 60.12	326.27 ± 48.27	375.00 ± 83.18	301.36 ± 72.70	345.57 ± 100.34
Heart "	65.40 ± 10.94	68.85 ± 13.01	65.42 ± 12.23	69.05 ± 8.52	65.03 ± 7.63	67.30 ± 10.78	59.26 ± 9.63	65.79 ± 9.84
Lt. ventricle & sept. "	46.18 ± 10.63	51.42 ± 10.92	47.24 ± 9.94	50.18 ± 9.43	48.67 ± 6.07	51.05 ± 8.95	44.58 ± 5.98	50.11 ± 7.84
Rt. ventricle (gm)	19.22 ± 5.21	17.93 ± 3.04	18.18 ± 5.03	18.53 ± 3.55	16.36 ± 1.91	16.25 ± 3.50	14.67 ± 4.46	15.69 ± 3.36
Kidney "	54.56 ± 10.69	63.80 ± 8.59	50.64 ± 11.85	60.91 ± 13.21	58.63 ± 5.75	63.21 ± 8.17	51.50 ± 7.29	57.28 ± 8.90
right "	27.91 ± 5.87	33.37 ± 4.32	25.13 ± 7.52	29.10 ± 5.92	30.96 ± 2.33	32.23 ± 3.48	26.46 ± 3.48	29.23 ± 5.45
left "	26.27 ± 4.48	30.47 ± 4.72	29.48 ± 11.39	31.98 ± 6.46	27.45 ± 3.18	30.95 ± 4.84	26.47 ± 4.70	28.28 ± 4.36
Adrenal "	1.91 ± 0.68	1.66 ± 0.37	1.97 ± 0.73	1.78 ± 0.53	2.24 ± 1.86	1.76 ± 0.35	1.78 ± 0.53	1.71 ± 0.44
Spleen "	40.75 ± 13.21	54.40 ± 19.15	38.15 ± 10.94	40.77 ± 10.38	37.20 ± 14.34	44.50 ± 18.19	44.65 ± 17.28	35.43 ± 11.99
Thyroid "	0.72 ± 0.22	0.75 ± 0.22	0.66 ± 0.26	0.67 ± 0.18	0.68 ± 0.24	0.80 ± 0.42	0.74 ± 0.23	0.63 ± 0.28
Brain "	76.29 ± 5.79	73.13 ± 5.12	72.46 ± 5.74	75.26 ± 5.27	73.04 ± 7.71	72.83 ± 7.55	73.27 ± 4.51	73.04 ± 6.77

Portions of heart and kidney tissue were fixed in glutaraldehyde for possible electron microscopy. All other tissues for pathological examination were preserved in 10% buffered formalin. The tissues underwent two changes of formalin within a week of the time they were obtained to insure that all tissues were adequately fixed. Following fixation, representative sections of all tissues were cut, embedded in paraffin using standard techniques, sectioned and stained with H&E for histological examination. Additionally, bone samples were either frozen or preserved in 10% buffered formalin for later use in determining bone lead.

Results

The organ weights are given in Table 1 as percentage of body weights. Statistically, after correction for body weight, no treatment effect was noted for any of the organ weights; however, values for the right kidney were close to significant in the auto exhaust atmospheres as opposed to the non-auto exhaust atmospheres. The other values were unremarkable (1).

A number of mammary tumors were present in the animals. These were predominantly mixed cell tumors rather evenly distributed in the various treatment groups (Table 2). Fewer tumors appeared in the auto exhaust atmospheres (Table 3). Additional work with the mammary tumors is being done at Davis but is not expected to have any impact upon the auto exhaust study. It is an extension of a beagle mammary tumor study.

Another common non-pulmonary finding in old dogs was the presence of mucoidal degeneration of the heart valves. This appeared to be very common and usually both valves were

Table 2. The Frequency Distribution of Mammary Tumors for Beagles Exposed to Exhaust and Non-Exhaust Atmospheres

Atmosphere	With tumors	Without tumors	Total
CA	11	6	17
R	3	7	10
I	3	7	10
SO_x	5	3	8
$R + SO_x$	3	6	9
$I + SO_x$	5	6	11
$NO_L + NO_{2H}$	7	3	10
$NO_H + NO_{2L}$	6	4	10
Total	43	42	85

Table 3. The Frequency Distribution of Mammary Tumors for the Beagle Exposed to Different Atmospheres

Type of exhaust	With tumors	Without tumors	Total
Auto exhaust	14	26[a]	40
Non-auto exhaust	29	16	45
Total	43	42	85

[a] $P<0.05$

involved. The findings are presented in Table 4. Very severe degeneration with a moderate amount of calcification appeared to be the cause of the pathologic heart sound heard by this and other investigators.

Skin tumors of a non-lethal nature were noted in several animals with no predominance in any treatment group. There were nine papillary type tumors; three were inflammatory polyps, two were small hemangiomas, and the rest were papillomas (Table 5). Several animals were affected with alopecia of varying degrees but none were serious. Of the animals seen with alopecia, none had abnormal endocrine histology. Other common findings were splenic nodules (all hyperplastic) in eight animals and unilateral thyroid enlargement in two animals; the latter were histologically thyroid-adenomas, most likely of non-secretory type (see Chapter 3).

The major emphasis was placed upon the pulmonary tissue; fixation was accomplished by allowing the lungs to float on fixative of 30 cm H_2O pressure for 16 to 18 hours. The lungs were carefully tied off with a heavy silk string and volume measurements by fixative displacement were taken. Any previously recorded abnormalities were rechecked and the lungs were placed in fresh fixative. The details of the section preparation will be given in Chapter 12; however, the gross lesions were as follows:

1) Eight lungs failed to completely collapse to the normal extent when removed from the chest. These came from the following groups: 3/R; $3/NO_H + NO_{2L}$; $1/NO_L + NO_{2H}$; $1/SO_x$.

2) Six lungs had small, discrete, marginal zones of atelectasis.

3) One lung had incomplete extra divisions.

A summary of the pulmonary histologic lesions are shown in Table 6. Further evaluation of the lungs will be presented in Chapter 12. The lead analyses were carried out on six femurs, three from non-exhaust and three from exhaust treated animals. The results are shown in Table 7.

Table 4. Mucoidal Degeneration of A-V Valves

Both	Left	Right
27	23	1

Table 5. Skin Lesions and Papillomas

Skin lesions		Skin papillomas	
Papillomas	7	Papillomas	4
Hyperkeratosis	1	Hemangiomas	2
Localized alopecia	3	Inflammatory polyps	3
Hemangiomas	2		

Table 6. Lung Lesions (No grading is included)

	Number of lungs with lesions							
	CA	R	I	SO_x	$R + SO_x$	$I + SO_x$	$NO_L + NO_{2H}$	$NO_H + NO_{2L}$
Inflammatory foci	4	3	1	3	1	2	1	1
Focal Type II hyperplasia	—	—	—	—	—	—	1	1
Increased thickness of alveolar walls and scar formation	1	1	1	2	1	3	1	5
Severe emphysema	—	1	—	1	—	—	2	4
Other lesions	2	2	—	1	2	1	1	—

Table 7. Lead Analysis of Femurs from Dogs (Beagles) Exposed to Chronic Auto Exhaust[a]

	Exposure	Dog #	Femur cortical (µg/gm)	Femur proximal end (µg/gm)
	SO_x	74R18	2.1828	0.9186
	NO_x	74R16	1.2822	2.7816
	CA	74R66	2.2858	2.1843
Average:			1.92	1.96
	I	74R12	4.5237	2.9724
	$I + SO_x$	74R41	2.9742	7.3799
	R	74R55	5.5346	4.7517
Average:			4.34	5.03

[a] Preliminary work on bone lead levels in six dogs. This work was done in Cincinnati and is indicative of bone lead level in exhaust versus non-exhaust animals.

Discussion

There were no unexpected non-pulmonary findings in the dogs. Dr. Hyde and I collaborated in the work on the lungs, which at this time is complete. Therefore the discussion of the pulmonary lesions will be left for Chapter 12.

For the last 5 years these animals have been extensively investigated and clinical disorders were always promptly evaluated and treated within the allowable regimen (see Chapter 3). One additional study (2) reported earlier showed a high incidence of hip displasia in these animals.

Only a very small number of animals were tested for bone lead. A higher bone lead level was seen in the exhaust as compared to non-exhaust treated animals (Table 7). Further data are not available at this time; however since all animals were essentially always treated equally, the additional lead component must be due to the exhaust lead. The lead levels in the exhaust atmosphere were discussed earlier in Chapter 1, and a more detailed description has been presented by Barkley *et al.* (3).

The cardiac valvular lesions are common in older dogs (4) and are usually seen in both valves or in the tricuspid valve. In this study the valvular lesions were more prevalent in the bicuspid valve. Only a few of these lesions were severe enough to be of clinical importance (5). Although mammary tumors appeared to be common in these dogs and some were diagnosed as adenocarcinomas, no animal showed any signs of tumor metastasis.

References

1. Andersen, A.C. 1970. Beagle: As an experimental dog. Iowa State University Press, Ames, Iowa.
2. Morgan, J.P. 1964. Hip displasia in the beagle: A radiographic study. J. Am. Vet. Med. Assoc. 164 (5): 496-498.
3. Barkley, N.P., K.A. Busch, W.L. Crider, and M. Malanchuk. 1972. The concentration of lead in automobile exposure chambers. Am. Indust. Hyg. Assoc. J. 33: 678.
4. Smith, H.A., T.C. Jones and R.D. Hunt. Veterinary pathology, 4th ed. Published by Lea and Febiger.
5. Bloch, W.N., Jr., T.R. Lewis, K.A. Busch, J.G. Orthoefer, and J.F. Stara. 1972. Cardiovascular status of female beagles exposed to air pollutants. Arch. Environ. Health 24: 342-353.

Asilomar Conference Discussion

Orthoefer: There are a lot of data on the dogs, kidney, liver, and all other organs, but really there are few lesions that are relatable back to the treatments. Practically all kidneys have hypercellular glomeruli. The animals have a lot of age-related lesions as distinguished from treatment-related lesions.

Dungworth: We have time for questions, but let's leave the pulmonary-related changes until after Dallas Hyde has presented his findings, and then we can take all the pulmonary changes from the three together. If there are questions on the non-pulmonary, let's have them now.

Orthoefer: The reason I presented this mucoidal degeneration of heart valves was because I knew there was going to be some question about the abnormal heart sounds and the cardiovascular work. These are the heart lesions, most of which are common in older dogs. Everybody that has listened to the heart of these dogs from about 1967 on has pointed out abnormal heart sounds in some dogs. The only thing it can be related to is the mucoid degeneration of the heart valves. It occurs as dogs get older. I wanted to point out that we didn't have any real malignant tumors of the skin. There are other lesions, some enlarged thyroids. In fact, there were two thyroid-adenomas, but the blood chemistry data did not indicate these were secreting or functional in any way. I did not include all that because we just can't relate it to treatment.

Dungworth: The cardiac lesions in aging dogs have been well documented by Patterson and Detweiler. I don't think there is any question about them.

Albert: What about the aorta? Were there arteriosclerotic lesions in the aortas?

Orthoefer: I made sections of all the aortas and I've still got them. I've looked at them, but have not done any special stains. The unstained aortas really looked fairly normal to me. Possibly we should do some special fat stains on whole mounts, or maybe we could contract it out. Grossly, there were no changes in the aorta.

Albert: You don't see any arteriosclerosis?

Orthoefer: No.

Dungworth: You do not generally see gross lesions in dogs unless they have diabetes or hypothyroidism.

Thurlbeck: In Table 1 that you showed of the lung lesions, would those be grade 3 emphysema?

Orthoefer: Yes, they would be a grade 3.

Thurlbeck: Using this as a point of reference, speaking in general terms in grade 3, you could say that given a lesion of this severity in man, particularly on the gross, it would be arguable with another pathologist whether emphysema was or was not present.

Dugnworth: We made that point in the discussion of our paper. It is mild in comparison to emphysema as usually described for man.

Orthoefer: Actually, in dogs, you would rarely see the human counterpart occurring unless you had very severe pulmonary function changes.

Dungworth: You can sometimes see severe lesions in old dogs. They tend to occur in subpleural airspaces first, however; here we are talking about comparing treatment versus control animals.

Kleinerman: If you had to grade whole lungs in a manner such as Dr. Thurlbeck or other pathologists looking at human lungs have described, would you have been able to detect emphysema on the gross cross section?

Orthoefer: Possibly. One thing I felt might be significant that is mentioned in the paper under the gross lesions is that some of these lungs had a doughy consistency. I thought it was significant in that it represented trapped air; however, it was not as consistent as I would like to have seen it.

Dungworth: I think we have to be careful in answering that question hastily. If we took a thick section from the control lung and a thick section from one of the NO_2-high and looked at them side by side, I believe one would say yes, there is a difference there, but it is only mild if judged by human standards.

Orthoefer: Actually, you can tell the presence of dilation of airspaces on the slide by just holding up the histologic slide and looking at it. I thought maybe you were speaking for the gross lung.

Kleinerman: That means all nine histologic slides, if you just pick one up at random from each case, you could tell it has dilation of the airspaces, or emphysema, if you compare it to a control slide?

Orthoefer: No, not on a 0 and 3 basis, but yes if it had severe emphysema as compared to a non-affected animal.

Kleinerman: Or you've got a slide of lung, a cross section, and you are looking at the mean size of the airspaces throughout the lung. Can you tell if there is emphysema in the slide?

Dungworth: In the worst affected, yes, but it was still mild as compared to human emphysema.

Orthoefer: I think Dr. Hyde's presentation is going to be important because you can visualize the lesions by SEM and it is more dramatic than the histologic lesion.

Dungworth: This point is addressed directly in Chapter 12. We don't say the lesions are severe, and the worst lesions we saw are mild relative to human emphysema in its usually noted spectrum. On that, we are clear and must accept it; however, it is unequivocally emphysema.

Kleinerman: Were there other significant findings as far as your grading scheme was concerned?

Orthoefer: There was pigment in the lungs but it's not severe. I graded the pigments on whether they were present. In fact, it was as bad in the controls as the treatment groups. One thing that was somewhat significant was the mucous glands, and I think Dr. Tyler has plans to pursue the quantitation study.

Dungworth: In his paper, Dr. Stephens mentioned "occasional evidence of exaggerated goblet cell activation and evidence of perivascular fibrosis." This is an impression that we obtained, but we are not in a position to quantitate it at the moment. We have to apply quantitative methods and that is in the process of being submitted for funding. So we remain sitting on the fence with regard to how much bronchial gland or mucous activity there is and so on, which I think is appropriate until we have more information.

Bhatnagar: Dr. Stephens, you mentioned that there were increases in the endoplasmic reticulum in many different types of cells.

Stephens: Only the type II cells.

Bhatnagar: Only in the type II cells of the exposed animals?

Stephens: Yes, but I would have to say that it occurs in a small number of type II cells found mainly in the proximal alveoli.

Bhatnagar: That would imply they are making a large amount of whatever type II cells make.

Stephens: That might be the case. That would be an assumption, of course.

Question: Did you say that you excised the lobe, weighed it, put it in fixative, and displaced the fixative to get the specific gravity?

Orthoefer: No. What I said was that the whole right lung, after it was preserved (fixed at 30 cm H_2O pressure, and expanded to its maximum), was measured using the displacement method of finding the volume of the right lung.

Question: And what did this tell you?

Orthoefer: It just tells you the volume of the right lung. The right lung is approximately 59% of the total lung volume.

Albert: In the animals that had the lobectomy was there any difference in the weight of the remaining lung?

Orthoefer: I did not weigh those lungs because the lobe was missing. Actually, the weighing started after the 14 lobectomized animals were done. Another point I would like to clarify is there were only 14 when the sacrifice began, because one of the lobectomized animals had died in California. Dr. Stephens mentioned six controls, and only had five. One of the animals he had as a control was a practice animal and it was lobectomized first before any of the auto exhaust dogs. We wanted to see if the technique would work and this was included as a control animal. This extra animal was not an auto exhaust dog. It came from the colony in Colorado which was maintained by the AEC.

Stephens: I was aware of that.

Stara: There were originally three subgroups of five controls, five NO_2-high, and five raw exhaust that were lobectomized.

Question: Why were these selected?

Stara: At the time, we felt that these three subgroups probably could be expected to demonstrate the most distinct changes.

Albert: Well, the point I was driving at was whether or not there were any data on the ability of the remaining lung to enlarge.

Orthoefer: I don't have displacement data on these lungs. I think weight is available. I'd have to go back and look, but I know displacement values are *not* available.

Littlefield: Is there anything in the literature where the effects of very low levels of carbon monoxide have been evaluated for as long as this exposure has gone on? You've got a 5-year exposure of carbon monoxide. Has the brain been looked at very thoroughly?

Orthoefer: I've looked at sections of all the brains. What is it you would like to see? We have visual evoked response and spontaneous EEGs. Dr. Johnson of NIOSH and I collaborated on several studies when he worked at our facility. But remember, we don't have CO alone. You have to attribute changes to auto exhaust, not CO *per se*. The evoked potential and spontaneous EEG study was done by putting animals down with secostrin, and maintaining them with artificial respiration. Then we did spontaneous EEGs and the so-called visual evoked response using a strobe light.

Question: Would that be as sensitive as a pathological examination?

Orthoefer: I think probably more so, when you're talking about brain. A morphometric study could be done where a person could count cells in the cerebrum; however, I've not been able to dream up such a study yet.

Dungworth: That would be a mammoth undertaking to find out whether there was anything important.

Orthoefer: The organ weights were compared as percent of body weight. We compared each treatment group to controls and then compared the exhaust versus non-exhaust, and found nothing significant in regard to total organ size regarding the brain.

Littlefield: My original question was, I don't think there's anything in the literature where they have animals that have been exposed for that long to carbon monoxide.

Orthoefer: We do have effects on blood, hematocrit, carboxyhemoglobin, etc.

Littlefield: My point is, I don't think it's ever really been examined and this may be an opportunity.

Stara: As Dr. Orthoefer said in the beginning, some of these side studies represent an afterthought as the exposures progressed, such as the cardiovascular studies and the neurophysiologic evoked potential study.

Lewis: Every time we did another such project, it represented a relative hazard to the dogs, if it involved anaesthesia. You could lose the animals.

Stara: The problem always occurred: should we or shouldn't we make another set of observations, and perhaps lose another dog. The evoked potentials were done immediately after removal from the atmospheres using sedation.

Lewis: Do you mean hours or days? Do you mean they had significant carboxyhemoglobin levels when you did the evoked potentials?

Orthoefer: Do you have the date in the paper? This information could be obtained from a combination of Dr. Johnson's paper (Chapter 9) and the blood data I presented earlier. One could also look at a published reference about the blood rheology of these dogs.[1]

---

[1] *Block, W.M., Jr., S. Lassiter, J.F. Stara, and T.R. Lewis. 1973. Blood rheology of dogs chronically exposed to air pollutants. J. Toxicol. and Appl. Pharmacol. 25: 576.*

Lewis: I think it is the spontaneous data that hasn't been analyzed yet, but Dr. Johnson talked this over with me before I left, since we were working on many things. If you use the conventional analysis of variance, there was nothing significant, but if you have taken the t-test comparison where you tested every treatment back to the control, there was a response, and he couldn't identify what that was at that time.

Stara: That is exactly the problem in the treatment of some of the data. For example, the data reported here before were analyzed using the t-test. Ken Busch and You Yen Yang must help us to evaluate all the papers from this point, and consider either both approaches or the most appropriate method.

Hyde: Isn't the analysis of variance test of two parameters equivalent to a t-test?

Busch: Yes. The problem is that neither of those is exact in the case of a categorical response. You don't have time for a 10-minute statistical summary. You enumerate all possible outcomes under the assumption that controls and test groups are merely random selections from a total group that is in fact unaffected by the exposures. An exact test would be to enumerate all possible ways these animals could have been subdivided at random, given that a certain number were destined to turn out in a certain way due to their inherent biological variability. Then you calculate this difference between rank means and note all possible values that this statistic could assume, cut off the upper 5% of the rank values, and if your sample value appears in the upper 5% you say this can't be a random result — it's so unlikely under a randomization. Therefore, we conclude that it wasn't random.

Orthoefer: Is that the ANOVA test?

Busch: No, that's a test I originated. This is an exact enumeration test based on hypothesis testing and basic statistical decision theory. You can only do it if you have a big computer and are willing to write the computer program to do it. Some of these nonparametric tests are made up of tables for all possible combinations. If you're willing to go to the trouble, even with a big table with nine categories, you could write a program and do it exactly.

Stara: Dr. Johnson has analyzed the data and he's responsible. I don't feel at liberty to change anything at this point.

Busch: If you find extreme significance, at the 1% or 0.1% level, with the t-test, then that's probably good enough. If you get approximately 5 or 10%, I wouldn't hang my hat on it, but I would check it by doing more precise calculations.

Stara: Gentlemen, let's proceed to the next paper.

12. MORPHOMETRIC AND MORPHOLOGIC EVALUATION OF PULMONARY LESIONS IN BEAGLE DOGS CHRONICALLY EXPOSED TO HIGH AMBIENT LEVELS OF AIR POLLUTANTS[1]

D. Hyde, J.G. Orthoefer, D. Dungworth, W. Tyler, R. Carter and H. Lum

One hundred and four (104) beagle dogs comprising one control and seven treatment groups were exposed 16 hours daily for 68 months to filtered air, raw or photochemically reacted auto exhaust, oxides of sulfur or nitrogen, or their combinations. After a further 32 to 36 months in clean air, morphologic examination of lungs by light microscopy, scanning electron microscopy, and transmission electron microscopy revealed two important exposure-related lesions. There were enlargement of air spaces in proximal acinar regions, with and without increases in the number and size of interalveolar pores, and hyperplasia of nonciliated bronchiolar cells. Proximal enlargement of air spaces was most severe, both subjectively and morphometrically, in those dogs exposed to oxides of nitrogen, oxides of sulfur, or oxides of sulfur with photochemically reacted auto exhaust. In contrast, hyperplasia of nonciliated bronchiolar cells was most severe in dogs exposed to raw auto exhaust alone or with oxides of sulfur. The air space enlargement and hyperplasia of bronchiolar epithelium correlated with functional impairment reported as occurring in these dogs. Foci of ciliary loss with and without squamous metaplasia were occasionally observed in the trachea and bronchi.

The observations indicate that enlargement of proximal acinar air spaces with some loss of interalveolar septa can develop in the absence of alveolar fenestrations. The persistent nature of bronchiolar cell proliferations in such circumstances was also demonstrated. Two major toxicologic implications are (1) the production of permanent lung damage by much lower concentrations of pollutants than previously reported and (2) the apparent lack of additive or synergistic effects between oxidant gases and sulfur oxides.

Epidemiologic studies have convincingly demonstrated that severe industrial smogs associated with high levels of sulfur compounds and particulates cause increased morbidity and mortality, particularly among people with chronic pulmonary or cardiac disease (7, 28, 44). These same reviews reveal that there is less convincing epidemiologic evidence linking increased incidence of disease with severe photochemical smog, whose principal components are ozone and other oxidant gases.

Many toxicologic studies have been conducted on animals in attempts to define more precisely the nature of effects likely to be caused by the common ambient air pollutants (4, 14, 21). Most have been for short exposure periods and at concentrations of individual pollutants greatly in excess of those ever encountered in urban smog. The purpose of this paper is to describe and comment on morphometric and morphologic findings derived from a study in which groups of beagle dogs were exposed for more than 5 years to a variety of polluted atmospheres of varying complexity and at concentrations reached in severe episodes of air

---

[1] *This paper was first published in 1978 in Laboratory Investigation, Vol. 38, No. 4, p. 455. It is reprinted here by permission of the U.S.-Canadian Division of the International Academy of Pathology.*

pollution. The pulmonary lesions present when the dogs were examined nearly 3 years after the end of exposures correlate well with abnormalities in cardiopulmonary function described elsewhere (references 9, 34, and 50 and J.R. Gillespie, J.D. Berry, Y. Yang, L.L. White, and J.F. Stara, personal communication).

Materials and Methods

One hundred and four (104) female beagle dogs were randomly assigned to exposure chambers which contained four dogs each (25). All of the dogs were from a single purebred colony and were approximately 6 months of age at the beginning of the exposure. One group of 20 control dogs (five chambers) was exposed to CBR (chemical, biologic, and radiologic) filtered air. The remaining dogs were divided into seven groups of 12 (three chambers), each of which was exposed 16 hours daily for 68 months to one of the following pollutants or pollutant combinations: raw (nonirradiated) auto exhaust (R); irradiated auto exhaust (I); oxides of sulfur (SO_x); R + SO_x; I + SO_x; high nitrogen dioxide (NO_2-high) plus low nitric oxide, and low NO_2 plus high nitric oxide (NO-high). Concentrations of the pollutants are given in Table 1. Various physiologic parameters were determined before, during, and after exposure (references 9, 34, and 50 and J.R. Gillespie *et al.*, personal communication). After exposure ended, the dogs were kept indoors in clean ambient air for 32 to 36 months before necropsy at Davis, California. In nearby Sacramento, California, the oxidant level averaged 0.05 ppm during the 3-year period (11). The dogs were maintained as an isolated colony and were under close clinical observation during the entire period of study. At no time was there evidence of infection or overt respiratory disease. During the 8 years of the experiment, 19 dogs died, mostly as a result of anesthetic procedures used for the cardiopulmonary function studies or from bite wounds received during fighting. Deaths occurred in all groups, and it was not possible to attribute direct causal relationship to the exposure atmospheres. We did not have access to 14 additional dogs which had right middle lobes removed surgically at the end of exposure (five dogs each from the I and NO_2-high groups and four dogs from the control group), hence 71 dogs were available at necropsy for this study (Table 1).

From 32 to 36 months after cessation of exposure, the dogs were weighed, measured, and killed by exsanguination after deep surgical anesthesia using sodium pentobarbital. The lungs, trachea, and attached structures were removed from the thorax *in toto* and trimmed of extraneous tissue (heart and mediastinum). Subsequently, the lungs were weighed, the left primary bronchus was ligated and the left lung was separated for biochemical analysis. The trachea was cannulated and the right lung was fixed in the normal dorsoventral orientation by intratracheal perfusion with cacodylate-buffered glutaraldehyde-paraformaldehyde at 30 cm. fixative pressure for 16 to 18 hours. The volume of the right lung was then measured by fixative displacement for subsequent morphometric evaluation. The lungs were stored in fresh fixative until they were blocked for microscopic examination.

Blocks of tissue (2 by 3 by 1 cm) for histologic examination were taken from nine different sites of each right lung of all dogs (Figure 1). Paraffin sections, 7 μm thick, were cut and routinely stained with hematoxylin and eosin.

The paraffin sections were used for stereologic evaluation. A linear correction factor for the shrinkage of the fixed tissue due to processing (p) was calculated by dividing the square root of the multiplied linear measurements of the sectioning face of the fixed tissue blocks by the

Table 1. Measured Pollutant Concentrations to Which Dogs Were Exposed 16 Hours a Day for 68 Months[a]

Atmosphere	No. of dogs[b]	Pollutant (mg/m^3)						
		CO	HC (as CH_4)	NO_2	NO	O_x (as O_3)	SO_2	H_2SO_4[c]
Control air (CA)	12							
Nonirradiated auto exhaust (R)	10	112.1 ± 11.5	18.0 ± 2.9	0.09 ± 0.04	1.78 ± 0.52			
Irradiated auto exhaust (I)	5	108.6 ± 22.5	15.6 ± 4.0	1.77 ± 0.68	0.23 ± 0.36	0.39 ± 0.18		
SO_2 + H_2SO_4 (SO_x)[d]	8						1.10 ± 0.57	0.09 ± 0.04
Nonirradiated auto exhaust + SO_2 + H_2SO_4 (R + SO_x)[d]	9	113.1 ± 15.9	17.9 ± 2.8	0.09 ± 0.06	1.86 ± 0.54		1.27 ± 0.61	0.09 ± 0.04
Irradiated auto exhaust + SO_2 + H_2SO_4 (I + SO_x)[d]	11	109.0 ± 22.8	15.6 ± 3.9	1.68 ± 0.68	0.23 ± 0.36	0.39 ± 0.16	1.10 ± 0.56	0.11 ± 0.04
Nitrogen oxides, 1 (NO_2-high)	6			1.21 ± 0.22	0.31 ± 0.08			
Nitrogen oxides, 2 (NO-high)	10			0.27 ± 0.62	2.05 ± 0.26			

[a] Exposure was from September 7, 1965, to May 7, 1971. Values are means ± 1 standard deviation; milligrams per cubic meter = parts per million molecular weight ÷ 24 at standard temperature and pressure (modified from Lewis *et al.* [34]).

[b] Number of dogs available at necropsy.

[c] Optical sizing indicated >90% of particles were <0.5 μm in diameter.

[d] x indicates oxides of sulfuric acid.

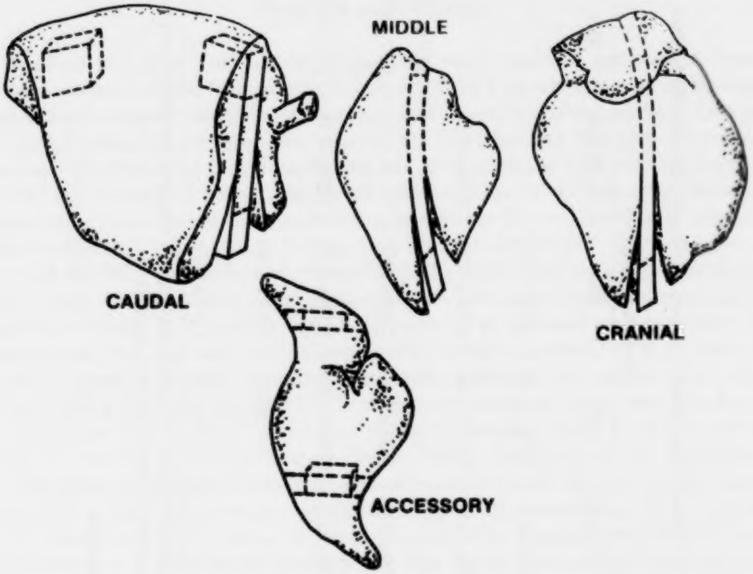


Figure 1
Cranial, middle, and caudal lobes of the canine right lung are depicted from the lateral view; the accessory lobe is depicted from the caudal view. Blocks of tissue for histologic evaluation were selected from indicated sites in transverse plane for cranial and middle lobes, sagittal plane for caudal lobe, and frontal plane for accessory lobe.

measurements on the processed sections (51). A screen magnification of X125 was used on an image analyzer to obtain the primary measurements (12). A guard frame of 100,000 picture points was used to avoid inaccurate measurements at the borders of the video screen, leaving a screen matrix of 500,000 picture points. The image analyzer, a microscope attached to a video system which is capable of measuring structures on the basis of density, has been shown to give stereologic values consistent with accepted manual methods in the dog lung (27). The image analyzer was set to detect the alveolar air spaces and the detection level was standardized by use of a preset level of detection on a specific field of a wire mesh grid.

Four random measurements were made at each of five stratified layers on each slide (47). Fields that contained large vessels or bronchi were rejected and other fields selected. The volumetric densities of alveolar airspace (V_{va}) and alveolar tissue (V_{vt}) (cm^3/cm^3) were estimated by the fractional area of points lying on their respective components. The volumetric density of alveolar tissue was corrected for the Holmes effect, the error introduced by finite section thickness (51). The volumetric correction coefficient ($K_{va} = 0.7$) was calculated from an alveolar diameter of 118 μm for the lungs of small dogs (45) and an interalveolar septal thickness of 10 μm. The volumetric density of alveolar airspace was adjusted accordingly.

The surface density of alveoli (S_{va}, m^2/cm^3) was calculated using the formula (52):

$$S_{va} = 4\, N_a / L_t \tag{1}$$

where L_t is the cumulative length of test lines (scanning lines on the video screen) and N_a is the number of intersections of test lines and interalveolar septa. Alveolar surface density was corrected for the effect of shrinkage of the fixed tissue due to processing by multiplying S_{va} by (p^2/p^3 or $1/p$) (51).

The internal surface area of the unfixed right lung (S_{ar}, m^2) was calculated using the formula (51):

$$S_{ar} = S_{va} \cdot (V_{Lr} \cdot V_{vp} P_p^2 f^2) \tag{2}$$

where V_{Lr} is the displaced volume of the processed right lung (volume of fixed right lung/p^3), V_{vp} is the volumetric density of parenchymal tissue estimated by point count of gross lung slices (51), p^2 is a factor for converting areas of processed to fixed tissue, and f^2 is a factor for converting areas of fixed to fresh tissue. The alveolar surface density (S_{va}) used in this formula is the uncorrected value. The linear correction factor for shrinkage due to fixation (f) was determined for each dog from a modification of the formula used by Thurlbeck (47):

$$f = \left(\frac{[TLC + W_L]\, 0.59}{V_{Lr}} \right)^{1/3} \tag{3}$$

where W_L is the weight of the lung and TLC is total lung capacity determined by a body plethysmograph (J. R. Gillespie *et al.*, personal communication). W_L was used as an estimate of tissue volume. The value of 0.59 is an estimate for the population average of the ratio of the right lung capacity to total lung capacity in normal dogs (13).

The sample means and standard errors of the morphometric parameters, W_B (body weight), W_L, TLC, V_{Lr}, S_{va}, S_{ar}, V_{vt}, V_{vp}, f, and p, were recorded for each group (Table 2). One-way analyses of variance (46) were used to compare the eight exposure groups for each of the morphometric parameters above and for internal surface area normalized by body weight (S_{ar}/W_b). In addition to the simultaneous comparison of the eight group means, each of the seven treatment group means was individually compared to the control group mean using Dunnett's method of multiple comparison (46). Further analyses of the alveolar surface density (S_{va}) were performed to compare the eight exposure atmospheres within each of the nine sample regions in the right lung (Figure 1) and to compare the dorsal regions to the ventral regions within each group.

Each sample region was also examined histologically and graded, without knowledge of treatment group, for three types of lesions commonly observed in old dogs (Table 3) (43). These lesions were not evaluated morphometrically. The sample regions were examined

Table 2. Morphometric Parameters for Dog Lungs from Control and Exposed Groups[a]

Group:	C	R	I	SO_x	$R + SO_x$	$I + SO_x$	NO_2-high	NO-high
No. of dogs:	12	10	5	8	9	11	6	10
W_B (kg)	9.08	10.24	9.15	9.24	9.61	9.89	9.06	9.40
	± 0.24	± 0.47	± 0.83	± 0.47	± 0.37	± 0.38	± 0.39	± 0.37
W_L (gm)	100.5	119.9[d]	111.6	121.9[d]	114.6	112.7	108.5	108.8
	± 3.7	± 5.3	± 11.7	± 4.7	± 6.9	± 3.2	± 5.8	± 4.1
TLC (cm^3)	1095	1374[d]	1234	1446[d]	1277	1272	1342[d]	1262
	± 64	± 69	± 90	± 51	± 80	± 55	± 97	± 51
V_{Lr} (cm^3)	424	500	529[d]	512[d]	466	497	525[d]	479
	± 25	± 31	± 32	± 23	± 28	± 17	± 28	± 21
S_{Va} (m^2/cm^3)[b]	0.0279	0.0279	0.0267	0.0254	0.0280	0.0257	0.0229[d]	0.0258
	± 0.0013	± 0.0013	± 0.0021	± 0.0012	± 0.0014	± 0.0009	± 0.0019	± 0.0008
S_{ar} (m^2)[c]	14.85	18.24	16.47	17.29	16.89	15.71	14.27	15.85
	± 0.81	± 1.49	± 2.08	± 1.00	± 1.39	± 0.73	± 1.30	± 0.68
V_{Vt} (cm^3/cm^3)	0.1541	0.1398	0.1303	0.1326	0.1562	0.1331	0.1426	0.1395
	± 0.0078	± 0.0036	± 0.0063	± 0.0060	± 0.0092	± 0.0043	± 0.0116	± 0.0071
V_{Vp} (cm^3/cm^3)	0.888	0.896	0.888	0.892	0.888	0.884	0.857[d]	0.898
	± 0.003	± 0.004	± 0.007	± 0.007	± 0.006	± 0.006	± 0.002	± 0.005
f	1.1835	1.2104	1.1459	1.2207	1.2086	1.1795	1.1770	1.1964
	± 0.014	± 0.017	± 0.028	± 0.023	± 0.014	± 0.0128	± 0.0272	± 0.0200
p	1.2170	1.1840	1.1700	1.1960	1.2060	1.2030	1.2270	1.2140
	± 0.0120	± 0.0120	± 0.0170	± 0.0170	± 0.0100	± 0.0210	± 0.0150	± 0.0160

[a] Values are group means ± 1 standard error of the mean. See text for explanation of morphometric parameters.

[b] Corrected for shrinkage due to processing.

[c] Corrected for shrinkage due to processing and fixation.

[d] Significantly different from the control group ($P<0.05$, Dunnett's multiple comparison test).

independently by two investigators who reached the same conclusions. For each lesion an average grade (grades were averaged over the nine regions) was computed for each dog. The dog averages were then used in the Kruskal-Wallis one-way analysis of variance (46) to test for differences between the eight groups for each lesion. Individual comparisons of the treatment groups to the control group were made using the Mann-Whitney U test (46).

Specimens were taken from each right lung for scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Tissue blocks (10 by 10 by 5 mm) were taken from dorsal and ventral regions of the trachea 2 to 3 cm cranial to its bifurcation, the ventrolateral wall of the right primary bronchus between the lobar bronchi of the middle and caudal lobes, and the dorsal and ventral regions of the right cranial and caudal lobes adjacent to those regions depicted in Figure 1. In the dorsal region of the right caudal lobe, tissue blocks were taken from the more caudal site. From each of those four blocks from the right cranial and caudal lobes, two specific sample sites were selected using a dissecting microscope. One sample contained segmental and subsegmental bronchi, while the other contained acini (terminal bronchioles, respiratory bronchioles, and adjacent alveolar parenchyma). This gave a total of 11 sample sites from each lung for SEM and TEM examination. One airway within each sample was bisected longitudinally using an alcohol-cleaned razor blade. One-half of each bisected airway and parenchyma was processed for SEM by dehydration in a graded series of ethanol followed by amyl acetate which was exchanged for liquid CO_2 in a critical point drying apparatus. The dried tissue was mounted on aluminum stubs and coated with carbon followed by gold-palladium in a high vacuum evaporator. An ETEC autoscan SEM was used to examine the specimens.

The complementary halves of specimens examined by SEM were processed for TEM using a modification of the large epoxy-embedded block technique (35). Sections (1 μm) were cut,

Table 3. Grading System for Selected Pulmonary Histologic Lesions

Lesions	Grade	Explanation
Pigment and dust accumulation	0	No significant pigment.
	1	Minimal amounts of pigment, usually within macrophages adjacent to smooth muscle of alveolar ducts and small airways.
	2	Clusters of pigment-laden macrophages in interstitium adjacent to vessels and airways, sometimes associated with recognizable lymphatics.
	3	Large accumulations of pigment-laden cells in perivascular and peribronchiolar sites.
Bronchial gland activity	0	Few or rare, inactive.
	1	Few or rare, active.
	2	Several, active.
	3	Many, active.
Calcification of bronchial cartilage	0	No calcification.
	1	Minimal calcification.
	2	Calcification in most of cartilage in section.

mounted on glass slides, and stained with methylene blue, azure II, and basic fuchsin. After histologic examination of these slides, specific areas from these blocks were excised, attached to the end of Beem capsules with epoxy resin, and sectioned for TEM. The thin sections were stained with uranyl acetate and lead citrate. In order to enhance the correlation between light microscopy, SEM, and TEM, the SEM blocks with significant lesions were reembedded for light microscopy and TEM observation of the same lesions. A Zeiss 10 electron microscope was used to examine the specimens.

Each SEM block of tissue was graded without knowledge of treatment group according to those lesions easily evaluated by the SEM but difficult to grade histologically (Table 4). Determination of the criteria for the grading system was based on examination of material by two investigators; tabulation of the data was done by one of them.

For each dog the average grade of the trachea, right primary bronchus, and segmental and subsegmental bronchi sites was recorded for lesions numbered 1 and 2. The dog averages were used to compare the eight exposure groups by the Kruskal-Wallis one-way analysis of variance method (46). Individual comparisons of overall, dorsal, and ventral regions of treatment to control group regions were made using the Mann-Whitney U test (46). Similarly, the average grades of lesions numbered 3 and 4 of overall, dorsal, and ventral regions were calculated in order to compare all groups and each treatment to the control group.

Table 4. Grading System for Selected Pulmonary Lesions Evaluated by SEM

Lesions	Grade	Explanation
Ciliary loss without squamous metaplasia[a,b]	0	No ciliary loss.
	1	<2 sites[c] of ciliary loss.
	2	2-5 sites.
	3	>5 sites.
Ciliary loss with squamous metaplasia[a,b]	0	No squamous metaplasia.
	1	<2 sites of squamous metaplasia.
	2	2-5 sites.
	3	>5 sites.
Nonciliated bronchiolar[d] cell hyperplasia	0	No hyperplasia.
	1	Hyperplasia in terminal bronchiole only.
	2	Hyperplasia in terminal bronchiole and respiratory bronchiole.
	3	Hyperplasia in terminal bronchiole, respiratory bronchiole, and alveolar duct.
Interalveolar pores[a,d]	0	Few small interalveolar pores.
	1	Many small interalveolar pores.
	2	Many small and few large interalveolar pores.

[a] Per SEM tissue specimen.

[b] Trachea and bronchi.

[c] Site = area > 100 μm x 50 μm.

[d] Distal airways.

Results

No significant gross lesions were observed. Eight lungs failed to collapse to the normal extent on removal from the chest. Three of these were from the R group, three from the NO-high, one from the NO_2-high, and one from the SO_x group. Six lungs had small, discrete, marginal zones of atelectasis. This had no significant relationship to specific treatment group or to subsequently discovered histologic lesions.

Morphologic interpretations of pulmonary lesions were the result of correlative examination of the same airways and adjacent parenchyma by light microscopy, SEM, and TEM, whereas quantitative evaluations of relative magnitude of involvement among the various groups of dogs were based on SEM and light microscopy. The normal histologic and ultrastructural morphology of the dog lung has been described by several investigators (16, 22, 37) and our observations are in general agreement with theirs. Only those anatomical structures that are particularly relevant to the lesions we observed in this study will be described. Major exposure-related lesions were within pulmonary acini (Figure 2a). They consisted of proximal acinary air space enlargement, with and without increases in number and size of interalveolar pores, and nonciliated bronchiolar cell hyperplasia. These will be described in detail. Inconsequential histologic lesions, which were as common in lungs of control animals as they were in exposed dogs, were rare minute foci of chronic pneumonia or small granulomas. The latter have usually been interpreted as being caused by migration of parasitic larvae (6).

The analysis of the graded severity of three types of pulmonary histologic lesions commonly observed in old dogs (Table 3) showed no significant differences between treatment and control groups.

The analysis of variance for the morphometric parameters, body weight (W_B), right lung volume (V_{Lr}), alveolar surface density (S_{va}), internal surface area of the right lung (S_{ar}), internal surface area of the right lung normalized by body weight (S_{ar}/W_B), fixation shrinkage (f), and processing shrinkage (p), showed no significant differences between the eight exposure groups. The analysis of lung weight (W_L) and volumetric density of alveolar tissue (V_{vt}) revealed nearly significant differences between the group means ($P = 0.085$ and 0.090, respectively). For the remaining parameters, total lung capacity (TLC) and volumetric density of parenchymal tissue (V_{vp}), the analyses of variance showed significant differences between the exposure atmospheres ($P = 0.0175$ and $P = 0.0003$, respectively).

Dunnett's multiple comparison test revealed no significant differences between the seven treatment group means and the control group mean for the parameters W_B, S_{ar}, V_{vt}, f, p, and S_{ar}/W_B. For the parameters S_{va} and V_{vp}, Dunnett's test showed the NO_2-high group mean to be significantly smaller than the control group mean. For the parameter W_L, the R and SO_x group means were significantly larger than the control group mean.

The average TLC for the R, SO_x, and NO_2-high groups was significantly larger than the average TLC for the control group. For the volume of the fixed right lung the averages of the SO_x, NO_2-high, and I groups were significantly larger than the control group. The results of the analyses of variance and multiple comparisons for each of the morphometric parameters are given in Table 5.

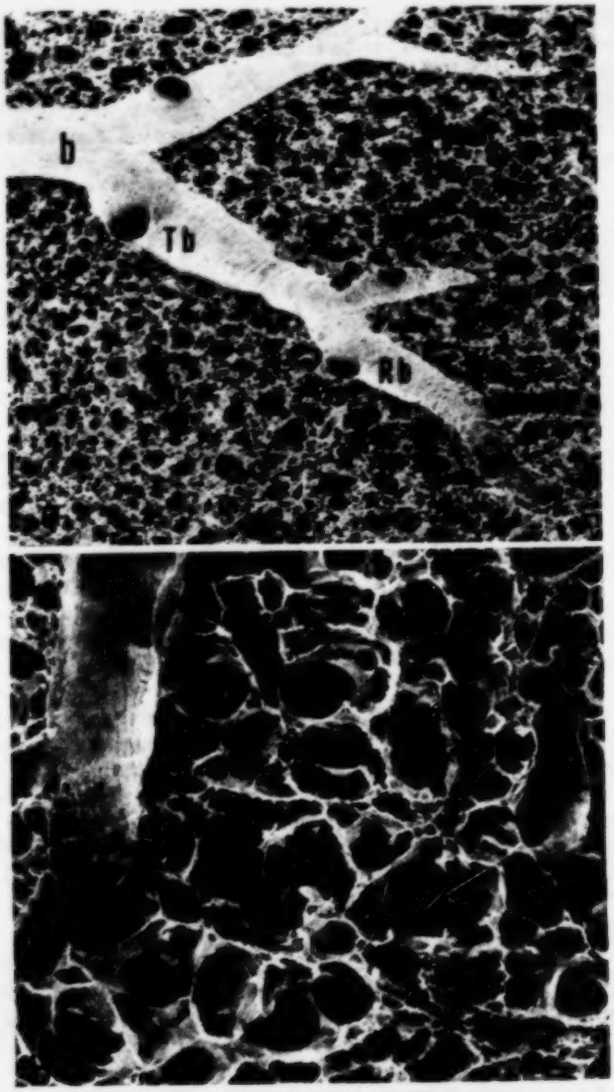


Figure 2.
a. Pulmonary acinus from a control dog originates from a distal bronchus (b) and consists of a terminal bronchiole (Tb) with a nonuniform branching pattern of respiratory bronchioles (Rb) and alveolar ducts. b. Lung from the NO_2-high group of dogs at the same magnification illustrates distal air space enlargement and strands of tissue (arrow). X20.

Table 5. Summary of Results for Morphometric Parameters[a]

Parameter	Analysis of variance	Group significantly different from control[b]
W_B	NS[c]	None
W_L	P = 0.0850	R, SO_x
TLC	P = 0.0175	R, SO_x, NO_2-high
V_{Lr}	NS	SO_x, NO_2-high, I
S_{va}	NS	NO_2-high
S_{ar}	NS	None
S_{ar}/W_B	NS	None
V_{vt}	P = 0.0900	None
V_{vp}	P = 0.0003	NO_2-high
f	NS	None
p	NS	None

[a] See text for explanation of morphometric parameters.

[b] Groups significantly different from the control with more severe lesions ($P<0.05$, Dunnett's multiple comparison test).

[c] No significant differences between group means exist ($P>0.10$).

Further analyses of S_{va} showed no significant differences between control and exposure groups for all regions of the lung, except for the NO_2-high group. The cranial, caudal, and accessory lobes and the dorsal and ventral regions of all lobes were significantly decreased from controls in the NO_2-high group.

Air Space Enlargement

The degree of enlargement of alveolar air spaces, which was derived from alveolar surface density, was notably different in the NO_2-high, SO_x, I + SO_x, NO-high, and I groups in decreasing order of severity (Figure 3). In the SO_x, I + SO_x, NO-high, and I groups, acinar involvement appeared to be random and its proportion and degree varied considerably among lungs of dogs within groups. The range was from an occasionally affected acinus to approximately a 50% involvement in any one histologic or SEM sample. Variability was also present among lungs of dogs in the NO_2-high group, but there were samples in which all acini were affected (Figure 2b). No preferential distribution was observed in any lobar region.

Structurally, the degree of enlargement as observed by light microscopy (Figure 4) corresponds well with the morphometric ranking of decreased alveolar surface density among exposure groups (Figure 3). Air space enlargement was centered on respiratory bronchioles and alveolar ducts. In conjunction with these lesions, there was also apparent loss of interalveolar septa (Figures 4 and 5).

Scanning electron microscopy revealed the apparent loss of alveolar walls more clearly (Figure 5). An important feature of the air space enlargement was that in the lungs of most exposure groups it was not associated with fenestrations or prominent increases in number and size of pores in interalveolar septa (Figure 5). On the other hand, considerable increases in number and size of interalveolar pores were found in some lungs without any air space enlargement (Figure 6). The lack of correlation between development of air space enlargement and enlargement of interalveolar pores was further emphasized by the fact that when the latter occurred it was distributed throughout the alveolar parenchyma, rather than being

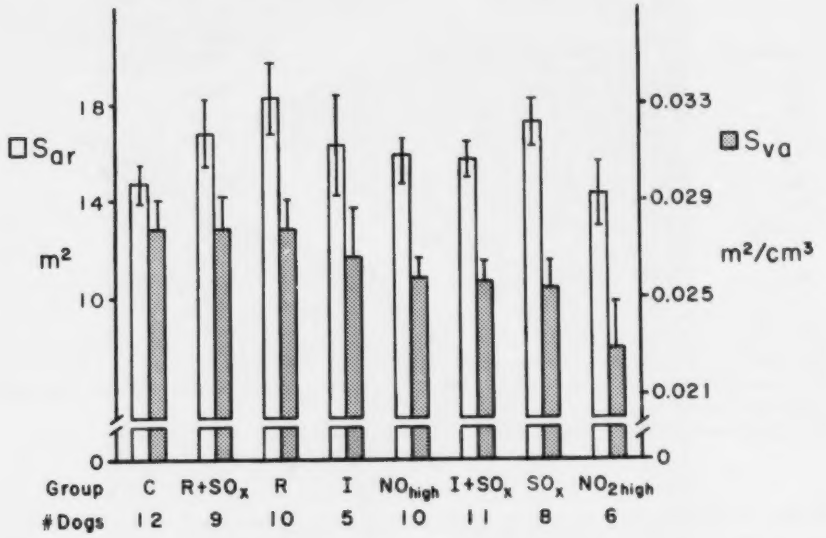


Figure 3
Comparison between the control and all other exposed groups by surface density (S_{va}, m^2/cm^3) and internal surface area of the right lung (S_{ar}, m^2). The control group to the left and all other exposed groups from left to right are plotted by decreases in S_{va}. Each bar represents the group mean ± 1 standard error. Significant differences from the control group are marked by an asterisk.

limited to proximal acinar regions as was the increase in size of air spaces. Although there were significant increases in frequency and size of interalveolar pores in most exposure groups as compared to the controls (Table 6), only in lungs of the most severely affected dogs in the NO_2-high and SO_x groups was there concurrence between air space enlargement and a striking increase in number and size of interalveolar pores (Figure 7).

Lesions in Conducting Airways
Predominant lesions in conducting airways were epithelial hyperplasia in bronchioles and foci of ciliary loss, with or without squamous metaplasia, in the trachea and bronchi. The relative severity of these lesions for the various treatment groups is significantly different from the control group as shown in Table 6.

The hyperplasia bronchiolar lesions consisted of discrete or confluent micronodular epithelial projections. The degree of hyperplasia was most pronounced in the R + SO_x and R groups of dogs where occasionally the hyperplasia was sufficient to cause partial occlusion of bronchiolar lumina (Figure 8). In least affected lungs, small hyperplasia lesions were observed at

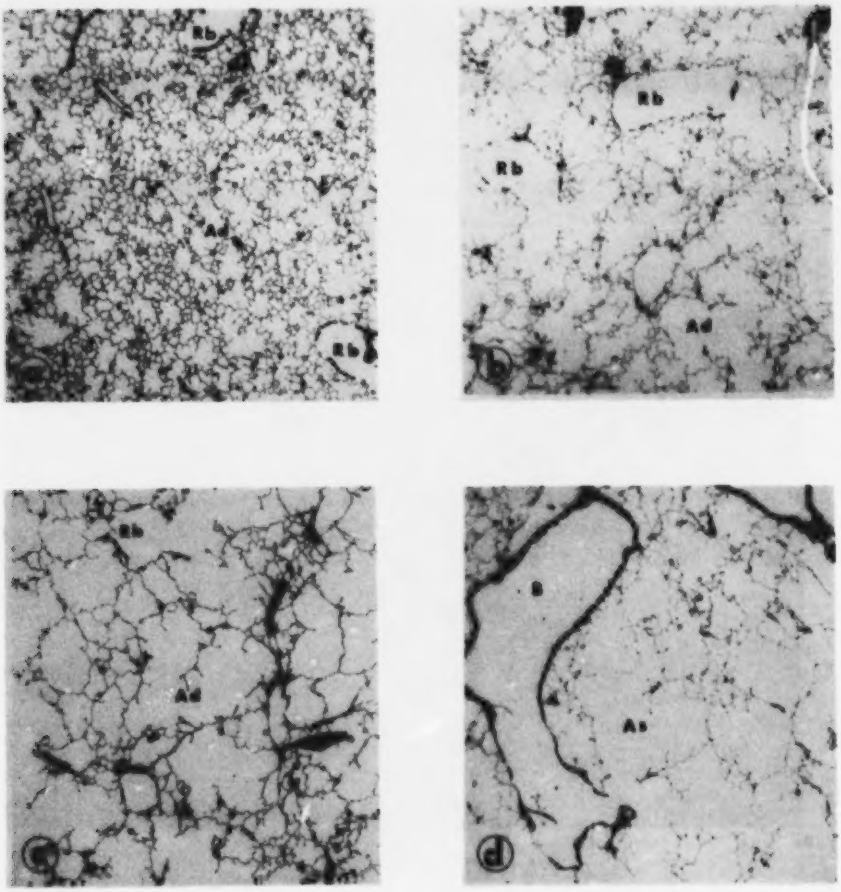


Figure 4
Comparison of distal airways and parenchyma in lungs from control and exposed groups. a. Numerous respiratory bronchioles (Rb) and alveolar ducts (Ad) are interspersed among alveoli in a lung from the the control group. b. Slight enlargement of respiratory bronchioles (Rb) and alveolar ducts (Ad) of a representative lung from the NO-high group. c. Mild enlargement and loss of interalveolar septa in alveolar ducts (Ad) in a representative lung from the SO_x group. d. Mild to severe enlargement and loss of interalveolar septa characteristic of a lung from the NO_2-high group. A respiratory bronchiole (Rb) is adjacent to a greatly enlarged alveolar duct (Ad). X16.

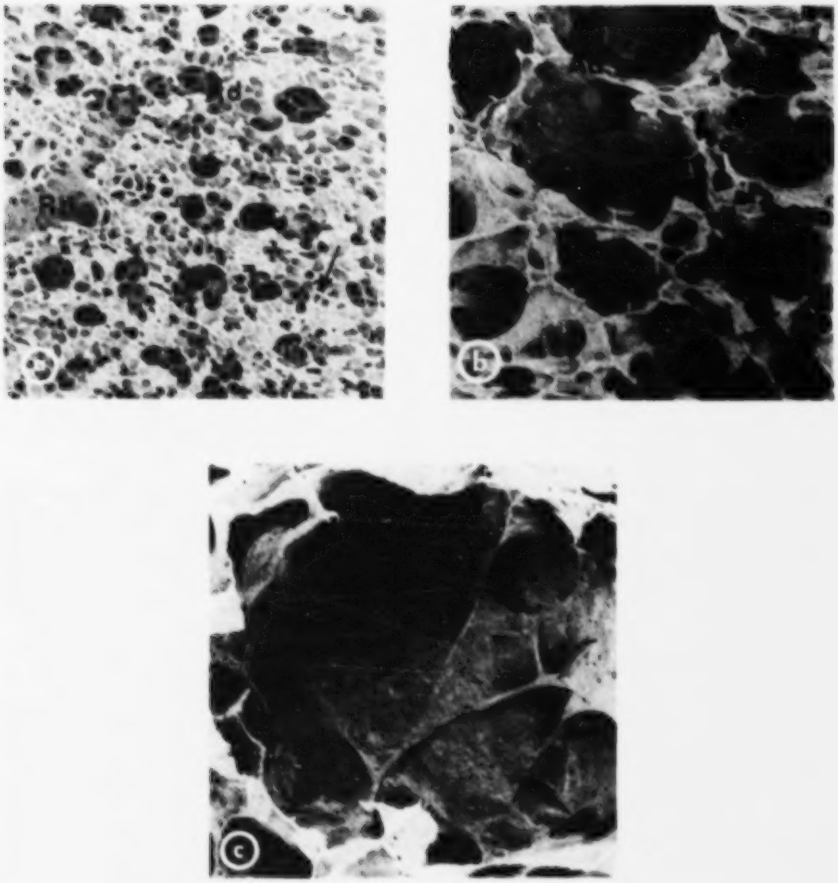


Figure 5
Comparison of distal airways in lungs from control and exposed dogs. a. Respiratory bronchiole (Rb) branches into alveolar ducts (Ad). Numerous alveoli and a moderate number of alveolar pores (arrow) are present in this lung of a control dog. b. Lung from the SO_x group of dogs at the same magnification illustrates alveolar duct (Ad) enlargement and loss of interalveolar septa. It is similar to the lesion in Figure 4c. c. Higher magnification in a region of b shows short interalveolar septa (black arrow) and shallow alveoli (a). Few interalveolar pores are seen (white arrow). Figure 5a and b, X50; c, X120.

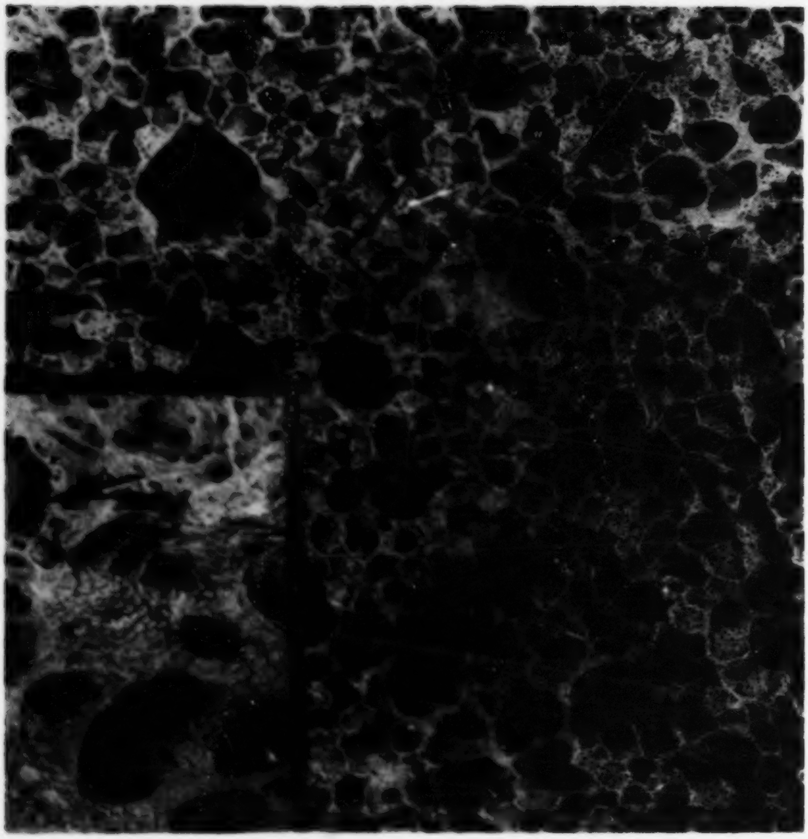


Figure 6
Respiratory bronchiole (Rb), alveolar ducts (Ad), and alveoli have the same conformation as controls despite widespread increase in interalveolar pores (inset, arrow) in a lung from the R group of dogs. X50; inset, X200.

the junction of the terminal bronchiole and the first-order respiratory bronchiole. Greater involvement consisted of more frequent and taller proliferations and their extension into all orders of respiratory bronchioles and the first-order alveolar duct.

The frequency of occurrence of hyperplastic lesions in acini was striking in the R group (65%) and R + SO_x group (42%) of dogs, whereas it was 20% or less in all other groups. Analyses of the average grades for the degree of severity of the hyperplastic lesion showed significant differences between exposure atmospheres (Table 6). The average grades for the R + SO_x,

Table 6. Summary of Results of Pulmonary Lesions Evaluated by SEM[a]

Lesions	Over-all differences between groups[b]	Groups significantly different from control[c,d]	Groups significantly different from control in dorsal region[d,e]	Groups significantly different from control in ventral region[d,e]
Ciliary loss without squamous metaplasia	NS[f]	SO_x, NO_2-high	None	None
Ciliary loss with squamous metaplasia	NS[f]	R + SO_x	None	None
Nonciliated bronchiolar cell hyperplasia	$P<0.005$	R + SO_x, R, I, NO_2-high, SO_x, I + SO_x	R, R + SO_x	R + SO_x, R, I, NO_2-high, NO-high
Interalveolar pores	$P<0.01$	NO-high, SO_x, R + SO_x, I + SO_x, R	NO-high, SO_x, R + SO_x, I + SO_x, R	NO-high, SO_x, R + SO_x, I + SO_x, R, NO_2-high

[a] For grading scheme see Table 4.

[b] Kruskal-Wallis test.

[c] Groups significantly different from the control group with more severe lesions ($P<0.05$, Mann-Whitney U test).

[d] Groups listed from most to least severe lesions.

[e] Groups significantly different from the control group with more severe lesions in the dorsal or ventral regions ($P<0.05$, Mann-Whitney U test).

[f] No significant differences found ($P>0.10$).

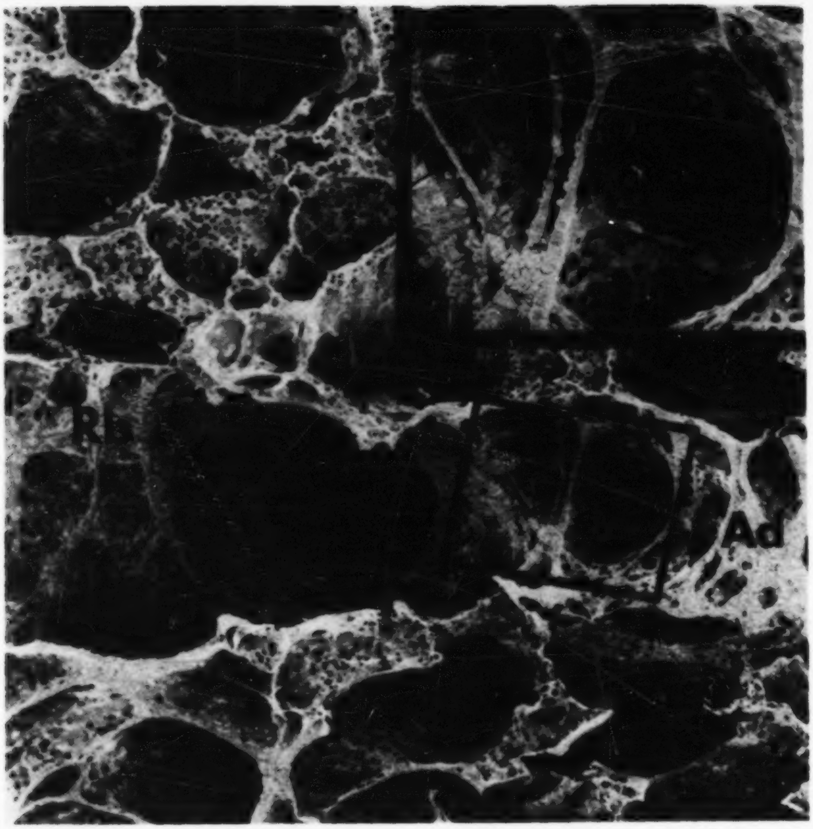


Figure 7
Junction between a distal respiratory bronchiole (Rb) and proximal alveolar duct (Ad) shows air space enlargement with numerous alveolar pores and fenestrations in a lung from the NO_2-high group of dogs. Fenestrations and trabeculae (arrow) are common in alveolar ducts (inset). X60; inset, X160.

R, I, NO_2-high, SO_x, and I + SO_x groups were significantly increased over the control group as listed from greatest to least severe. In the dorsal regions only the R + SO_x and R groups were significantly increased over dorsal regions in the controls, whereas the ventral regions of the R + SO_x, R, I, NO_2-high, and NO-high groups were significantly increased over the ventral regions of the controls. Within each group, however, no significant difference was observed between dorsal and ventral regions.

The hyperplastic nodules were predominantly composed of hypertrophic nonciliated bronchiolar cells (Figures 9 and 10). The nonciliated bronchiolar cells in exposed and control dogs

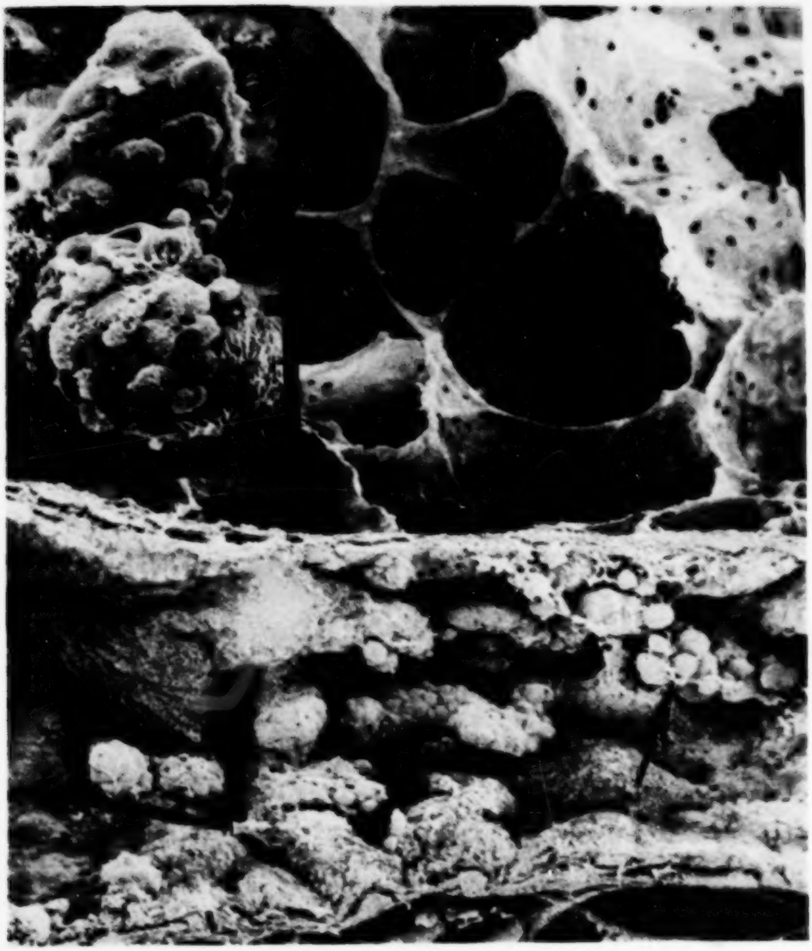


Figure 8
Irregular hyperplasia of nonciliated bronchiolar cells (inset) partially occludes the lumen of a terminal bronchiole in a lung from the R group of dogs. An aggregation of inflammatory cells (arrow) is seen in the distal region of the terminal bronchiole. X250; inset, X920.

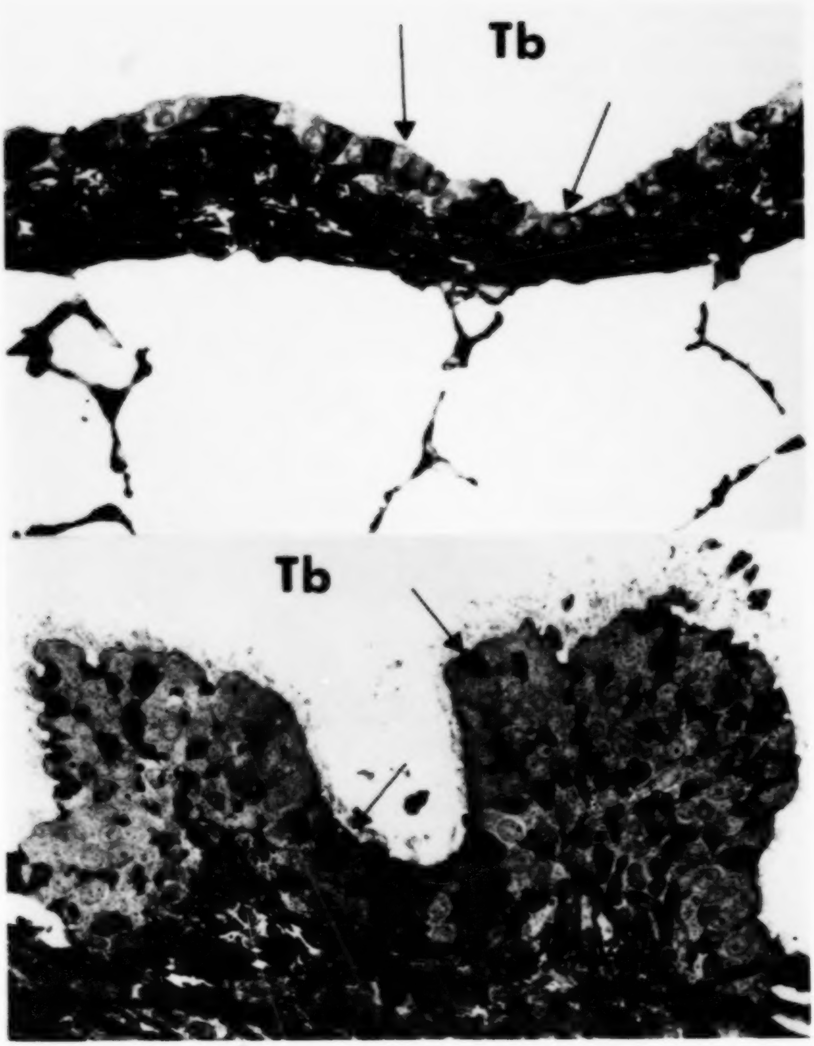


Figure 9
Comparison of terminal bronchiolar epithelium in lungs from control and exposed dogs. a. Epithelium of a terminal bronchiole (Tb) from the lung of a control dog has a mixture of ciliated (arrows) and nonciliated cells. b. Hyperplastic nodules in a terminal bronchiole (Tb) of a lung from the I group of dogs show a mixture of hypertrophied ciliated and nonciliated cells. Goblet cells (arrow) are also visible (1 μm Epon-embedded section). X370.

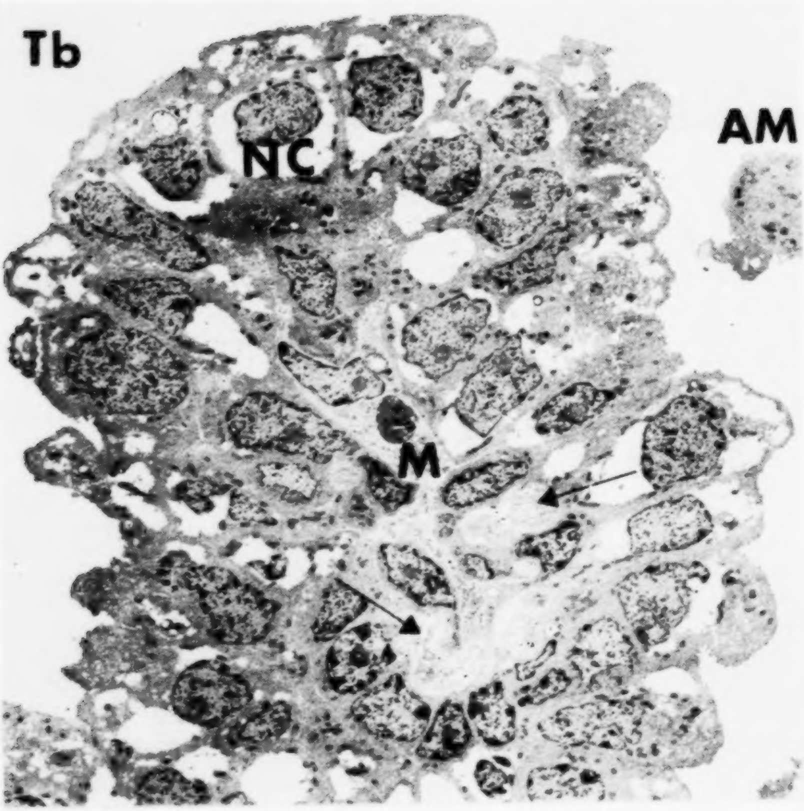


Figure 10
Hyperplastic nodule composed primarily of nonciliated cells protrudes into the lumen of a terminal bronchiole (Tb) in a lung from the I group of dogs. The clear areas in the nonciliated cells (NC) represent areas of fixative-leached glycogen. A macrophage (M) and segments of a connective tissue core (arrows) are also present. An alveolar macrophage (AM) is in the airway lumen. X1,600.

contained large clear areas, scattered lipid inclusions, a few dense staining mitochondria, an inconspicuous Golgi apparatus, sparse rough endoplasmic reticulum, and occasionally, accumulations of smooth endoplasmic reticulum in the apical region. In lungs of dogs (external controls) stored in fixative for short periods of time prior to TEM processing, the cytoplasm of nonciliated bronchiolar cells contained large amounts of glycogen. Since the lungs in this study were stored in fixative for more than a year before being processed for TEM, we believe the clear areas in nonciliated bronchiolar cells represent regions from which glycogen was leached during storage. In addition to the major component of hypertrophic nonciliated cells, ciliated cells and undifferentiated cells were also present in the large proliferations (Figure 11).

The principal lesions observed in trachea and bronchi of exposed dogs were foci of ciliary loss, with and without associated squamous metaplasia. These abnormalities were also rarely present in airways of control dogs. On subjective evaluation, there appeared to be a definite increase in size and frequency of these lesions over controls in at least some of the lungs of dogs in all exposure groups. Analysis of the grading showed significantly increased ciliary loss without squamous metaplasia in the NO_2-high and SO_x groups, whereas ciliary loss with squamous metaplasia was significantly increased in the R + SO_x group (Table 6).

In control dogs, the epithelial lining of trachea and bronchi consisted principally of ciliated cells and mucous-producing (goblet) cells. The cilia were shorter and less numerous on the dorsal membranous portion of the trachea that extends between the incomplete tracheal cartilages. In this location, ciliated cells, nonciliated cells (Figure 12), and openings of submucosal glands were easily observed with SEM. In the epithelium of the ventral and lateral regions of the trachea and of the bronchi, the cilia were more numerous and long enough to conceal most of the nonciliated cells and glandular openings from view with the SEM.

In exposed dogs, the distribution of foci of ciliary loss associated with squamous metaplasia differed from that of foci of ciliary loss alone. The foci of ciliary loss alone that were counted as significant were greater than 100 by 50 μm in size and were distributed randomly in all levels of airway. The cilia were usually completely absent from the surface epithelium, but some lesions had cells with long microvilli and a few short cilia (Figure 13). The composite cells were predominantly of the cuboidal mucus-secreting type. Basal cells were in their normal position and had no unusual distinguishing characteristics.

Foci of ciliary loss associated with squamous metaplasia were observed predominantly in the primary bronchus and the dorsal region of the trachea, but were also observed in the intrapulmonary bronchi in the NO_2-high, SO_x, and I + SO_x groups of dogs. Foci of ciliary loss associated with squamous metaplasia typically protruded into the airway lumen and were up to 500 μm in diameter (Figure 14). Sections from reembedded SEM blocks containing these lesions confirmed the nature of the squamous metaplasia (Figures 15 and 16). Other components observed in this type of lesion were cells with lucent to intermediate density inclusions, surface-ciliated cells with few cilia, cells beneath the surface with no cilia but with numerous basal bodies randomly distributed in the cytoplasm, and a variety of inflammatory cells. Cells adjacent to the basal lamina were often columnar in shape, and their basal borders often had cytoplasmic projections through the basal lamina into the lamina propria.

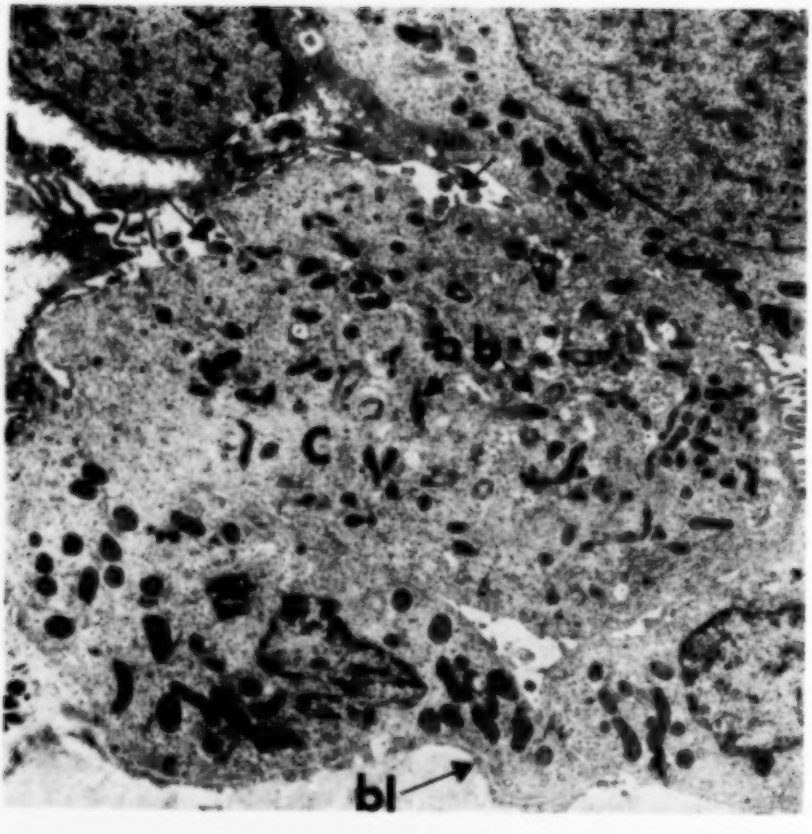


Figure 11
In a lung from the NO_2-high group of dogs, a ciliated cell (C) lies deep within a bronchiolar hyperplastic nodule, close to the basal lamina (bl). The cytoplasm has dispersed basal bodies (bb) and cilia are in the intercellular space (arrow). Serial sections showed cilia exiting from this cell. X6,700.

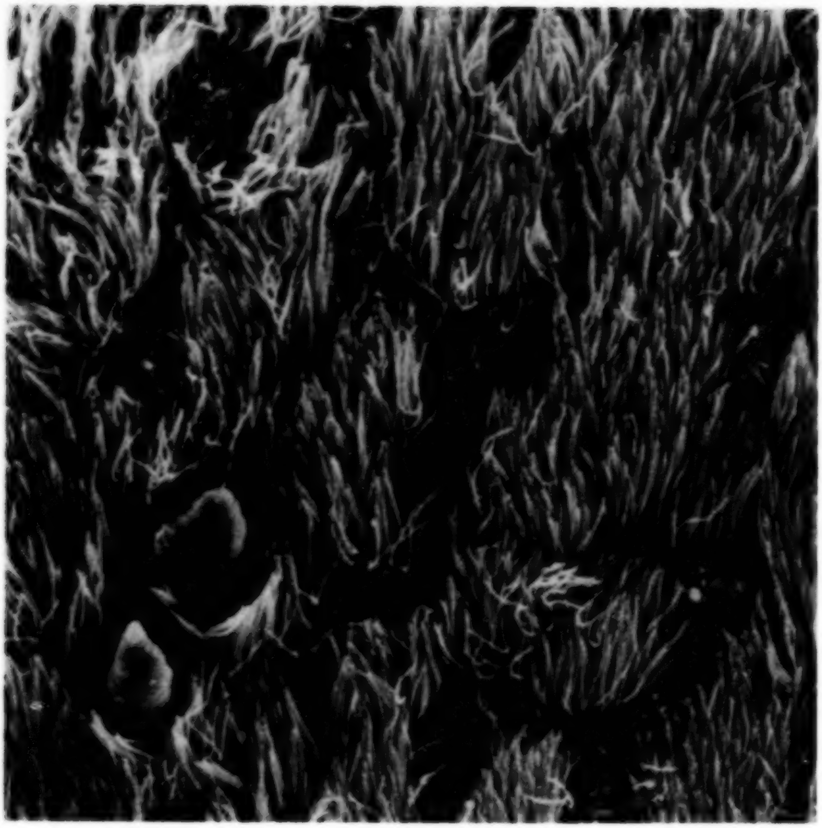


Figure 12
In the dorsal membranous portion of the trachea, the ciliated cells with short cilia (C) and long microvilli and the apical portion of goblet cells (G) are easily seen in a lung from a control dog. X2,000.

Two potentially important aspects of lesions in airways have not been satisfactorily evaluated. These are changes in the number and activity of mucous glands and goblet cells, and peribronchiolar fibrosis. Histologic examination provided equivocal evidence that these features were increased above controls in lungs of exposed dogs. Accurate comparisons of the activity of the mucous apparatus among the various treatment groups will be made morphometrically.

Discussion

The findings of air space enlargement and hyperplasia of nonciliated bronchiolar cells in lungs from beagles of most treatment groups 32 to 36 months after exposure ended have

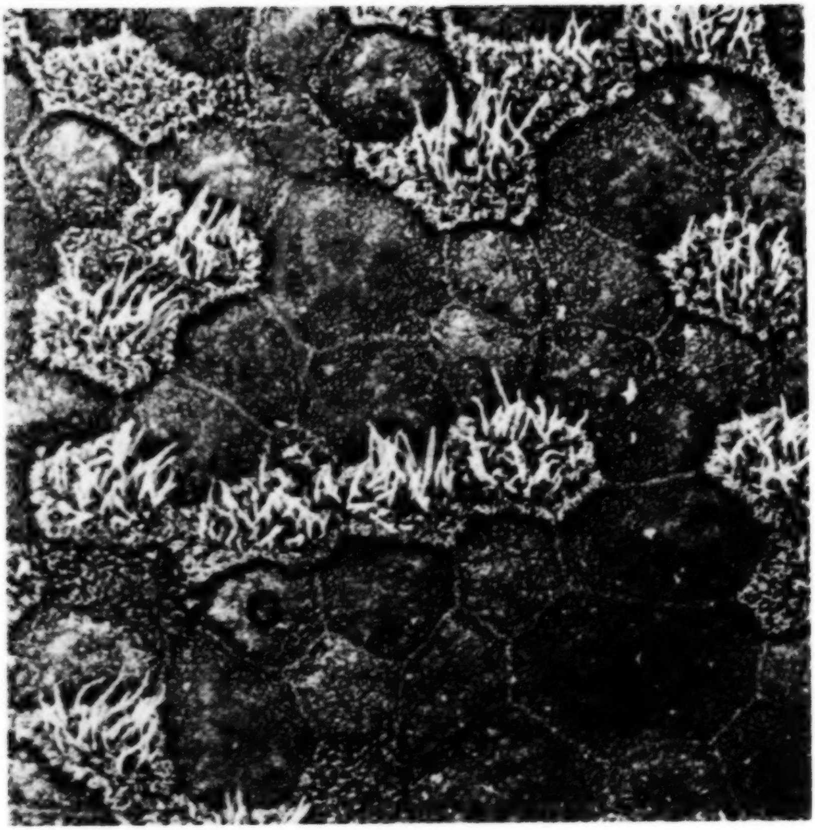


Figure 13
Focus of ciliary loss without squamous metaplasia in a primary bronchus from a lung in the NO_2-high group of dogs. Ciliated cells are reduced in number and have fewer cilia per cell. Goblet cells do not have apical projections (G), but ciliated cells have numerous microvilli (C). X2,350.

important implications with regard both to pathogenesis of such lesions and for the toxicology of air pollutants.

Morphologically, both histologic and SEM observations revealed that in lungs from five of the seven treatment groups there was appreciable enlargement of distal air spaces which was centered on respiratory bronchioles and alveolar ducts and was associated with apparent loss of interalveolar septa. These observations are supported by the morphometric decreases of alveolar surface density in the same groups (Figure 3). Since the enlargement occurred primarily in the region of respiratory bronchioles and alveolar ducts, as opposed to being

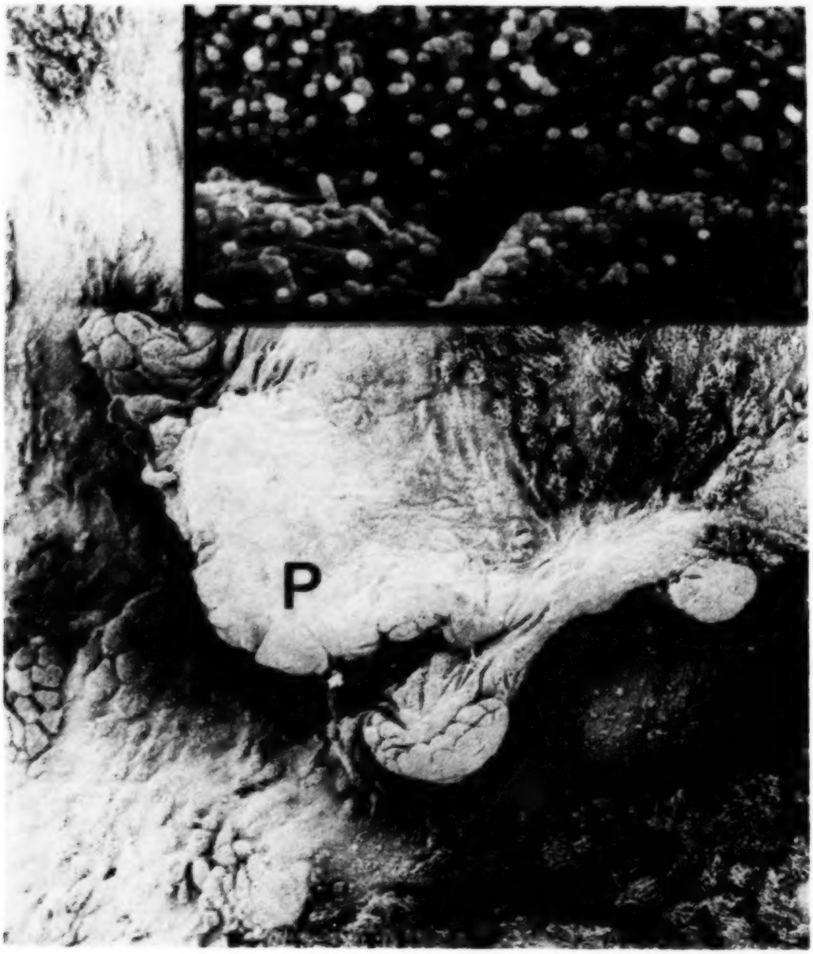


Figure 14
Focus of ciliary loss accompanied by squamous metaplasia is seen as a proliferative lesion (P) which protrudes into an airway lumen of a bronchus of a lung from the R group of dogs. The surface of the lesion has numerous microvilli and surface folds (inset). X310; inset, 6,550.

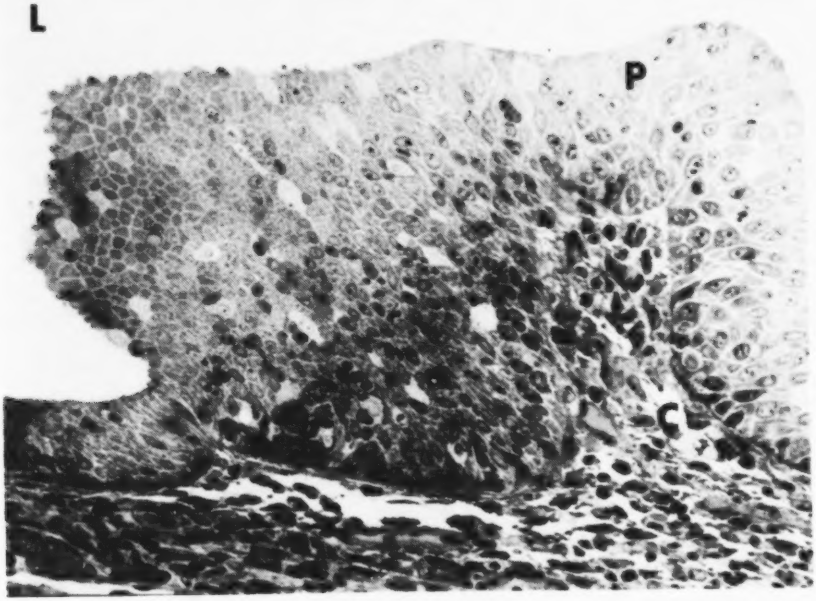


Figure 15
Section of a proliferative lesion (P) that protrudes into the lumen (L) of a distal bronchus in a lung from the NO_2-high group of dogs illustrating squamous metaplasia. At least five cells deep, the lesion has a connective tissue core (C) containing numerous mononuclear inflammatory cells (1 μm Epon-embedded section). X375.

distributed throughout the alveolar parenchyma, we consider it to be analogous to an incipient stage of human proximal acinar (centrilobular) emphysema (2). Although it was of mild degree compared to the clinically important cases of human emphysema (41), it was sufficient to cause abnormalities in pulmonary function. The two groups with the greatest decrease in alveolar surface density (NO_2-high and SO_x groups) (Figure 3) had higher than normal dynamic compliance at lower frequencies than the control group (J.R. Gillespie *et al.*, personal communication). The investigators interpreted this difference as evidence of decreased lung elasticity in those groups (J.R. Gillespie *et al.*, personal communication).

Since alveolar surface area increases directly with increases in lung volume (equation 2), the notable increases in the internal surface area of the right lung in most exposure groups as compared to controls (Figure 2) are primarily attributed to increases in lung volume (Table 2). The internal surface area of the right lung of dogs in the NO_2-high group was approximately the same as the control group (Figure 3), even though lung volume and lung weight were significantly increased in the NO_2-high group over controls (Table 5). It seems likely that the loss of alveolar tissue in the NO_2-high group (Figure 3) counterbalanced the increase in alveolar surface area that would be expected with a simple increase in lung volume.

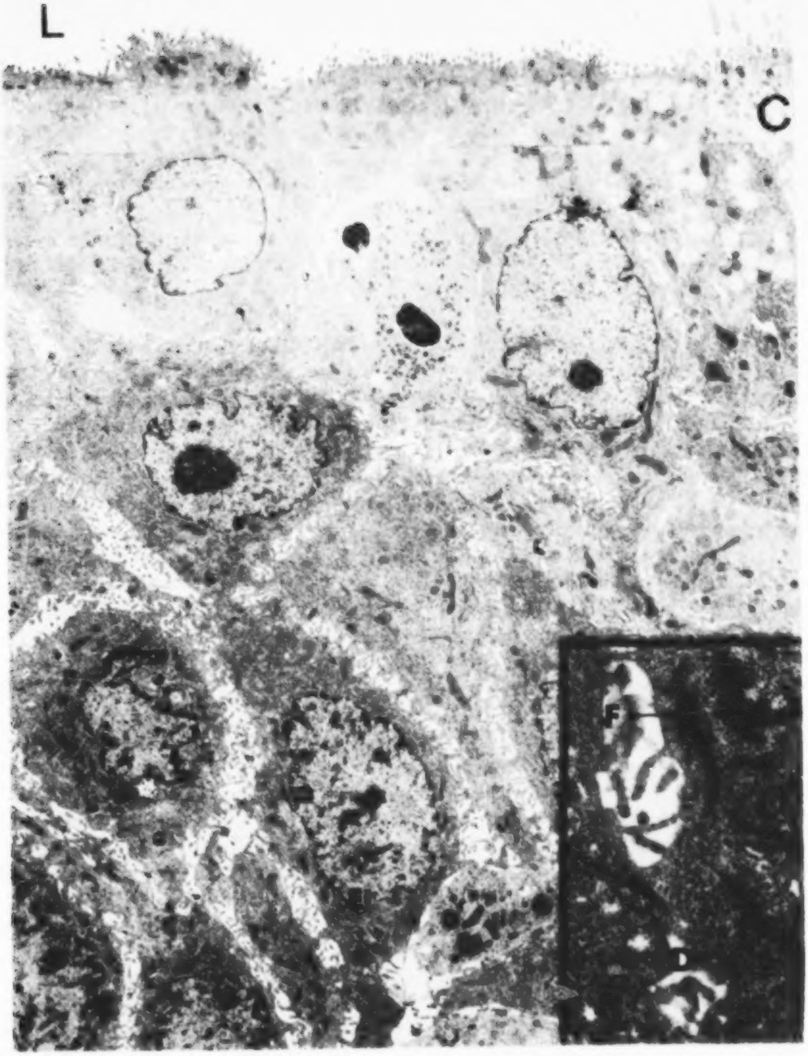


Figure 16
Thin section adjacent to the area seen in Figure 15. Columnar- to spindle-shaped cells with intracellular fibrils (F) and numerous intercellular processes connected by desmosomes (D) (inset) were the distinguishing characteristics of squamous metaplasia. A ciliated cell (c) sends cilia into the bronchial lumen (L). X3,150; inset, X14,250.

Comparison of our control group value for internal surface area of the right lung (14.85 m^2) with that predicted by body weight from a group of 22 mongrel and beagle dogs (18.63 m^2) (45), shows a lower than predicted value for our control group. We attribute the difference to our use of light microscopy, whereas Siegwart *et al.* (45) used electron microscopy. In a study from the same laboratory, it was shown that surface density increases proportionally to the optical resolution of the method of measurement (29). The increase in surface density by approximately 25% using electron microscopy (29) accounts for the difference in internal surface areas. The possibility that overinflation in some exposed groups accounted for the marked decreases in alveolar surface density seems very unlikely since there were no significant differences in the fixation factor (f) between control and exposed groups (Table 5). Hence we conclude that lung inflation and shrinkage due to fixation were not significantly different among any of the groups of beagles.

Two patterns of morphologic development of emphysema have been described. One is the progressive enlargement and coalescence of holes (fenestrations) in the walls of respiratory bronchioles and alveolar ducts, with progressive shortening of interalveolar septa (23). Kuhn and Tavassoli (32) have shown by SEM that the morphogenesis of elastase-induced emphysema in the hamster is of the latter pattern. It is generally believed that the increase in size of air spaces in human proximal acinar (centrilobular) emphysema is associated with fenestration (10, 32, 41, 42). SEM observations in this study indicate, at least in the dog, that proximal acinar enlargement of air spaces can develop in the absence of fenestration. In fact, this was the usual event. Increases in number and size of interalveolar pores, which appear to be the basis for large fenestrations (10, 42), when present were fairly evenly distributed throughout acini. Only in the most severely affected lungs of dogs in the NO_2-high and SO_x groups was there concurrence between enlargement of air spaces and exaggerated numbers and sizes of pores. Here strands of tissue considered to be hallmarks of human centrilobular emphysema were also present.

A further aspect of the alveolar lesions detected in lungs of these dogs deserves mention; namely that pulmonary function studies showed a gradual worsening of abnormalities associated with air space enlargement between the end of exposure and after 2 years of recovery in clean air (J.R. Gillespie *et al.*, personal communication). Although we cannot comment on the extent of morphologic changes over the same time period, the finding of increased levels of prolyl hydroxylase activity in lungs of most of the groups of dogs in absence of increases in total collagen content (39) suggests that there is persistent increase in collagen turnover. This accords with continued development of emphysema after a single administration of elastase to hamsters and the suggestion that abnormal mechanical stresses induced by the original insult might cause persistent rearrangement and hence progression of the lesion (32, 33). In the beagles, this would be superimposed on the enlargement of air spaces seen in control aging populations (27).

Hyperplasia of bronchiolar epithelial cells, predominantly of the nonciliated variety, was present to some degree in all groups of exposed dogs. We have not called nonciliated bronchiolar cells of the dog "Clara" cells because if the latter designation is to be useful it should be limited to cells with specific ultrastructural and functional characteristics, not used as a blanket term for all cells without cilia-lining bronchioles in all species. Since there are definite ultrastructural differences between nonciliated bronchiolar cells of dogs and Clara cells such as those studied in rodents (31), we prefer to use the more noncommittal term in our study of dogs until the situation is resolved. The bronchiolar proliferations had persisted despite removal of the original inciting insult approximately 3 years previously. Because we examined the bronchiolar lesions at one point in time, we cannot establish whether the proliferative nodules were slowly regressing or irreversible. The lengthy period since cessation of exposures and evidence from pulmonary function studies, particularly measurements of pulmonary resistance in the most severely affected group of dogs (R + SO_x), make it less likely that the proliferation was regressing. There was no evidence of atypical cellular features considered to be indicative of a premalignant change, but these proliferative lesions might conceivably be more responsive to carcinogenic stimuli than normal epithelium (15). A similar persistence of nodular hyperplasia of nonciliated bronchiolar (Clara) cells was reported in mice where proliferation caused by a 120-day exposure to ozone was still present after an additional 120 days in clean air. Other components of airway damage such as ciliary loss and metaplasia of tracheal epithelium resolved during the 120-day recovery period (40).

The ciliary loss and squamous metaplasia observed in trachea and bronchi appeared to be of minor importance. Speculations regarding reasons for their persistence parallel those made for bronchiolar cell hyperplasia. Squamous metaplasia in dogs has been caused by a wide variety of inhaled irritants such as ozone (19), sulfuric acid mist (1), sulfur dioxides (3), and cigarette smoke (5, 17), but only in the last named instance is there evidence that it might progress to squamous cell carcinoma (5). The R + SO_x group of dogs showed a significant increase in squamous metaplasia in comparison with controls (Table 6). Pulmonary resistance was also highest in the R + SO_x group, possibly in part due to increased airway turbulence associated with squamous metaplasia. It is of interest that the R + SO_x group showed the greatest severity of bronchiolar hyperplasia (Table 6) but no air space enlargement. The frequency of bronchiolar hyperplasia was also approximately fourfold that of squamous metaplasia in the R + SO_x group of dogs. Although total pulmonary resistance is usually considered to be principally an index of resistance to flow in larger airways (36), it has been reported that the diameter of bronchioles is the major determinant of pulmonary resistance in a study on a series of human postmortem lungs (38). If this is true in lungs of dogs it would clearly account for our findings. It is also possible that accompanying the bronchiolar lesions there was a degree of bronchial narrowing which we failed to appreciate.

Several important toxicologic inferences can be drawn from the results of this study, although assessment is made more difficult by the complexity of the exposure atmospheres and the fact that concentrations of only a few components of the raw or irradiated auto exhaust were measured. The production of some degree of irreversible damage in the lungs of all exposure groups at much lower pollutant concentrations than previously reported was the salient finding.

We are cognizant of no comparable studies in which the effects of automobile exhaust alone or with oxides of sulfur have been studied during or after long-term exposure.

The production of incipient emphysema by nitrogen oxides and irradiated exhaust, which contains both nitrogen oxides and ozone, is not surprising in view of previous reports that these gases can cause emphysematous lesions (8, 14, 18). The low concentration at which this occurred is surprising, however, because for nitrogen dioxide alone, for instance, the concentration required to produce moderately severe emphysema in rats is reported to be 28.75 mg/m^3 continuous exposure for the natural life span of a rat (18), whereas in this study 1.21 mg/m^3 of NO_2 and 0.31 mg/m^3 of the less injurious NO for 68 months at 16 hours a day produced what we considered to be mild but definite emphysema.

The presence of the air space enlargement in lungs of dogs exposed to sulfur oxides was unexpected because SO_2 has not been associated experimentally with its production. In the closest comparable study in which guinea pigs and monkeys were chronically exposed to fairly low concentrations of SO_2, H_2SO_4 mist, fly ash, or their mixtures (1), the authors concluded that the abnormalities detected were attributable to the H_2SO_4 at an approximate concentration of 1 mg/m^3. These abnormalities were minor amounts of epithelial erosion, goblet cell hyperplasia, squamous metaplasia in large airways, and hyperplasia of nonciliated bronchiolar cells. In our study, air space enlargement was caused by one-tenth the concentration of H_2SO_4 (0.09 mg/m^3) together with an amount of SO_2 (1.10 mg/m^3) comparable to that used in the guinea pigs and monkeys (1). The production of focal areas of emphysema in rodents and rabbits after acute experimental exposure to high levels of H_2SO_4 mist (49) further supports our observations.

The order of severity of hyperplasia of nonciliated bronchiolar cells for the various treatment groups was essentially the reverse of that for air space enlargement. This is to say, the hyperplastic lesions were most severe in the groups exposed to raw automobile exhaust, with and without addition of sulfur oxides. These groups were the only ones without some degree of air space enlargement (Figure 3). This can probably be explained by variation in chemical constituents of the exposure atmospheres. Raw exhaust contains polycyclic hydrocarbons which have been shown to cause carcinomas when painted on the skin of mice (26). It is of interest that experimental exposures of mice to ozonized gasoline (volatilized) increased the incidence of spontaneous pulmonary tumors and produced hyperplastic and metaplastic changes in the bronchial epithelium (30). It is probably polycyclic hydrocarbons that account for raw exhaust stimulating the greatest degree of bronchiolar epithelial proliferation. The lesser effect of irradiated exhaust could be accounted for by any photochemical reduction of the concentration of the hydrocarbons, as occurred in the case of methane, the one hydrocarbon measured. The other theoretical explanation for separation of bronchiolar epithelial hyperplasia and air space enlargement is that the bronchiolar hyperplasia in some fashion protected against the development of air space enlargement. We do not favor this latter hypothesis.

Since in smoggy environments exposure is to complex mixtures of pollutants, it is necessary to be cognizant of the degree of interaction that exists among components that up to now have usually been tested individually. Pulmonary function tests on human volunteers acutely exposed to SO_x and ozone, singly and in combination, have indicated a synergistic or potentiating effect of SO_2 on the acute bronchoconstrictive effects of ozone (24). A synergistic effect of the two gases in causing visible damage to foliage of various plants can often be demonstrated, although the effect on plant growth more often appears to be an additive one (48). In this study, comparison of the magnitude of chronic pulmonary damage caused by SO_x with that of irradiated auto exhaust + SO_x does not indicate either additive or synergistic effects of SO_x and oxidant gaseous components (NO_2 and O_3) present in the irradiated exhaust. A possible explanation for this is that aggregated particulate aerosols were present in chambers containing irradiated auto exhaust and adsorption of gaseous components on larger particles might decrease deep pulmonary penetration of the irritant gases.

A conclusion to be drawn with respect to the toxicology of air pollutants is that accurate experimental assessment of harmful effects of relevant concentrations requires prolonged exposures coupled with the most sensitive measurements of functional and structural disturbances.

References

1. Alarie, Y.C., A.A. Krumm, W.M. Busey, C.E. Ulrich, and R.J. Kantz, II. 1975. Long-term exposure to sulfur dioxide, sulfuric acid mist, fly ash, and their mixtures: results of studies in monkeys and guinea pigs. Arch. Environ. Health 30: 254.
2. American Thoracic Society. 1962. Chronic bronchitis, asthma, and pulmonary emphysema: statement by the Committee on Diagnostic Standards for Nontuberculous Respiratory Disease. Am. Rev. Respir. Dis. 85: 762.
3. Asmundsson, T., K.H. Kilburn, and W.N. McKenzie. 1973. Injury and metaplasia of airway cells due to SO_2. Lab. Invest. 29: 41.
4. Assembly of Life Sciences, National Academy of Sciences-National Research Council. 1973. Proceedings of the Conference on Health Effects of Air Pollutants. Washington, D.C., U.S. Government Printing Office.
5. Auerbach, O., E.C. Hammond, D. Kirman, and L. Garfinkel. 1970. Effects of cigarette smoking on dogs. II. Pulmonary neoplasms. Arch. Environ. Health 21: 754.
6. Barron, C.N., and L.Z. Saunders. 1966. Visceral larva migrans in the dog. Vet. Pathol. 3: 315.
7. Bates, D.V. 1972. Air pollutants and the human lung. Am. Rev. Respir. Dis. 105: 1.
8. Blair, W.H., M.C. Henry, and R. Ehrlich. 1969. Chronic toxicity of nitrogen dioxide. II. Effect on histopathology of lung tissue. Arch. Environ. Health 18: 186.
9. Bloch, W.N., Jr., T.R. Lewis, K.A. Busch, J.G. Orthoefer, and J.F. Stara. 1972. Cardiovascular status of female beagles exposed to air pollutants. Arch. Environ. Health 24: 342.
10. Boren, H.G. 1962. Alveolar fenestrae: relationship to the pathology and pathogenesis of pulmonary emphysema. Am. Rev. Respir. Dis. 85: 328.
11. California Air Resources Board. 1973. 10 year summary of California air quality data, 1963-1972, p. 146. Sacramento, California.
12. Cole, M. 1966. The metals research Quantimet. Microscope 15: 148.
13. Cree, E.M., J.R. Benfield, and H.K. Rasmussen. 1968. Differential lung diffusion capillary volume and compliance in dogs. J. Appl. Physiol. 25: 186.
14. Dungworth, D.L., C.E. Cross, J.R. Gillespie, and C.G. Plopper. 1975. The effects of ozone on animals. Pp. 29 ff. *in* J.S. Murphy and J.R. Orr, eds. Ozone chemistry and technology: A review of the literature 1961-1974. Philadelphia, Franklin Institute Press.
15. Farber, E. 1973. Carcinogenesis: cellular evolution as a unifying thread: presidential address. Cancer Res. 33: 2537.
16. Frasca, J.M., O. Auerbach, V.R. Parks, and J.D. Jamieson. 1968. Electron microscopic observations of the bronchial epithelium of dogs. I. Control dogs. Exp. Mol. Pathol. 9: 363.
17. Frasca, J.M., O. Auerbach, V.R. Parks, and J.D. Jamieson. 1968. Electron microscopic observations of the bronchial epithelium of dogs. II. Smoking dogs. Exp. Mol. Pathol. 9: 380.
18. Freeman, G., S.C. Crane, N.J. Furiosi, R.J. Stephens, M.J. Evans, and W.D. Moore. 1972. Covert reduction in ventilatory surface in rats during prolonged exposure to subacute nitrogen dioxide. Am. Rev. Respir. Dis. 106: 563.
19. Freeman, G., R.J. Stephens, D.L. Coffin, and J.F. Stara. 1973. Changes in dogs' lungs after long-term exposure to ozone: light and electron microscopy. Arch. Environ. Health 26: 209.
20. Deleted in proof.
21. Goldstein, E. 1975. Reevaluation of the United States air quality standard for nitrogen dioxide. Rev. Environ. Health 2: 5.

22. Groniowski, J., M. Walski, and W. Biczysko. 1972. Application of scanning electron microscopy for studies of the lung parenchyma. J. Utrastruct. Res. 38: 473.
23. Hartroft, W.S. 1945. The microscopic diagnosis of pulmonary emphysema. Am. J. Pathol. 21: 889.
24. Hazucha, M., and D.V. Bates. 1975. Combined effect of ozone and sulphur dioxide on human pulmonary function. Nature 257: 50.
25. Hinners, R.G., J.K. Burkart, and G.L. Contner. 1966. Animal exposure chambers in air pollution studies. Arch. Environ. Health 13: 609.
26. Hoffman, D., E. Theisz, and E.L. Wynder. 1965. Studies on the carcinogenicity of gasoline exhaust. J. Air Pollut. Control Assoc. 15: 162.
27. Hyde, D.M., N.E. Robinson, J.R. Gillespie, and W.S. Tyler. 1977. Morphometry of the distal airspaces in the lungs of aging dogs. J. Appl. Physiol. 43: 86.
28. Kavet, R.I., and J.D. Brain. 1974. Reaction of the lung to air pollutant exposure. Life Sci. 15: 849.
29. Keller, H.J., H.P. Friedli, P. Gehr, M. Bachofen, and E.R. Weibel. 1976. The effects of optical resolution on the estimation of stereological parameters. Proceedings of the Fourth International Congress for Stereology, National Bureau of Standards Publication 431: 409.
30. Kotin, P., H.L. Falk, and C.J. McCammon. 1958. III. The experimental induction of pulmonary tumors and changes in the respiratory epithelium in C57 BL mice following their exposure to an atmosphere of ozonized gasoline. Cancer 11: 473.
31. Kuhn, C., L.A. Callaway, and F.B. Askin. 1974. The formation of granules in the bronchiolar Clara cells of the rat. I. Electron microscopy. J. Ultrastruct. Res. 49: 387.
32. Kuhn, C., and F. Tavassoli. 1976. The scanning electron microscopy of elastase-induced emphysema: a comparison with emphysema in man. Lab. Invest. 34: 2.
33. Kuhn, C., S. Yu, M. Chraplyvy, H.E. Linder, and R.M. Senior. 1976. The induction of emphysema with elastase. II. Changes in connective tissue. Lab. Invest. 34: 372.
34. Lewis, T.R., W.J. Moorman, Y. Yang and J.F. Stara. 1974. Long-term exposure to auto exhaust and other pollutant mixtures. Arch. Environ. Health 29: 102.
35. Lowrie, P.M., and W.S. Tyler. 1973. Selection and preparation of specific tissue regions for TEM using large epoxy-embedded blocks. Pp. 324 ff. *in* C.J. Arceneaux, ed. Proceedings of the Thirty-First Annual EMSA Meeting. Baton Rouge, Claitor's Publishing Division.
36. Macklem, P.T., and J. Mead. 1967. Resistance of central and peripheral airways measured by a retrograde catheter. J. Appl. Physiol. 22: 395.
37. Martin, H.B. 1966. Respiratory bronchioles as the pathway for collateral ventilation. J. Appl. Physiol. 21: 1443.
38. Niewoehner, D.E., and J. Kleinerman. 1974. Morphologic basis of pulmonary resistance in the human lung and effects of aging. J. Appl. Physiol. 36: 412.
39. Orthoefer, J.G., R.S. Bhatnagar, A. Rahman, Y. Yang, S.D. Lee, and J.F. Stara. 1976. Collagen and prolyl hydroxylase levels in lungs of beagles exposed to air pollutants. Environ. Res. 11: 299.
40. Penha, P.D., and S. Werthamer. 1974. Pulmonary lesions induced by long-term exposure to ozone. II. Ultrastructure observations of proliferative and regressive lesions. Arch. Environ. Health 29: 282.
41. Pratt, P.C., and K.H. Kilburn. 1970. A modern concept of the emphysemas based on correlations of structure and function. Hum. Pathol. 1: 443.
42. Pump, K.K. 1974. Fenestrae in the alveolar membrane of the human lung. Chest 65: 431.

43. Robinson, N.E., and J.R. Gillespie. 1973. Morphologic features of the lungs of aging beagle dogs. Am. Rev. Respir. Dis. 108: 1192.
44. Severs, R.K. 1975. Air pollution and health. Tex. Rep. Biol. Med. 33: 45.
45. Siegwart, B.P., P. Gehr, J. Gil, and E.R. Weibel. 1971. Morphometric estimation of pulmonary diffusion capacity. IV. The normal dog lung. Respir. Physiol. 13: 141.
46. Steele, R., and J.H. Torrie. 1960. Principles and procedures of statistics, pp. 99, 111, 404, 406. New York, McGraw-Hill.
47. Thurlbeck, W.M. 1967. The internal surface area of nonemphysematous lungs. Am. Rev. Respir. Dis. 95: 765.
48. Tingey, D.T., and R.A. Reinert. 1975. The effect of ozone and sulfur dioxide singly and in combination on plant growth. Environ. Pollut. 9: 117.
49. Treon, J.F., F.R. Dutra, J. Cappel, H. Sigmon, and W. Younker. 1950. Toxicity of sulfuric acid mist. Arch. Industr. Hyg. Occup. Med. 2: 716.
50. Vaughn, T.R., Jr., L.F. Jennelle, and T.R. Lewis. 1969. Long-term exposure to low levels of air pollutants: effects on pulmonary function in the beagle. Arch. Environ. Health 19: 45.
51. Weibel, E.R. 1963. Morphometry of the human lung, pp. 14, 45. New York, Academic Press.
52. Weible, E.R., G.S. Kistler, and W.F. Scherle. 1966. Practical stereological methods for morphometric cytology. J. Cell Biol. 30: 23.

Asilomar Conference Discussion

Hyde: . . . Nonciliated bronchiolar cell hyperplasia again was graded by the level of severity. There was never a case observed where there was involvement in the respiratory bronchioles or first order alveolar duct where it did not involve the terminal bronchiole. Thus it was possible to reliably use this type of grading scheme.

Lewis: What's the definition of ciliary loss?

Hyde: It is where no cilia are present on the cells in the conducting airways.

Lewis: How do you know it's a ciliated cell? Is it a ciliated cell without cilia?

Hyde: This will be ciliary loss defined strictly from the surface morphology.

Lewis: I'm asking what the criterion is. How do you know you're not in an area where there aren't any cilia?

Hyde: In the normal lung, there are no areas that are not ciliated, essentially.

Lewis: Well, you must be talking of an area of a certain size. There are certainly tiny areas where there are no cilia.

Hyde: Yes, excuse me. The area has to be 50 microns by 100 microns in size or greater.

Lewis: I see. So if you have a zone that large with no cilia, then that's an area of ciliary loss.

Dungworth: Excluding the openings of glands.

Hyde: Another point is that some of the sites seen were larger than 2 to 3 mm in diameter, so they were quite extensive. Since there was an increase in alveolar pores in most of the exposed groups, we tried to give a ranking to them since it's very difficult to quantitate interalveolar pores. I used three levels. All the controls had a few small interalveolar pores, and when there were numerous interalveolar pores I gave the sample a grade 1. Where there were many with some large interalveolar pores, I gave the sample a grade 2. This was just to get a quantitative feel for if there was any possible association between interalveolar pores and the enlargement we appreciated with the stereology.

Kleinerman: This was the number of pores, but not size of pores?

Hyde: No, both are considered. In a grade 2 sample, large pores would be the size of two or three average size pores that might be joined together. There had to be a large open space that would be at least 20 to 30 microns in diameter before it would be defined as a large pore. I tried to avoid the word fenestrae.

Albert: Wouldn't it be better if you had some pictures that could serve as standards?

Hyde: That's the difficulty with presenting a table first, followed by micrographs.

Albert: No, I mean grading. If you had some reference standards, you could take a look at the lung and say it looks most like this particular picture or that one, and thus grade the severity of the damage to the alveoli.

Hyde: My categories are so broad that it had to be fairly well into a category before I gave it that grade. I went for the mean, and there's no problem looking at a few interalveolar pores. You immediately say this sample has a few interalveolar pores, or this stub has a few; this one has many; this one has a few and a few large ones. So the delineations of the categories are not that difficult. If they were more fine, then I'd say, yes, I need the micrographs, but I don't feel I did.

Thurlbeck: One normally refers to centrilobular (centriacinar) emphysema as dominantly or selectively involving respiratory bronchioles. My concept is that the lesion should be quite proximal and the alveoli distal to the emphysematous space should be normal. I recognize that a dog's anatomy is quite different from human anatomy, so it's very difficult to make an analogy. This lesion really looks like elastase emphysema. I know people argue what that is, and I'd rather just call it emphysema.

Hyde: I should state that the lesion is similar in appearance to elastase-induced emphysema that was recently reported on by Kuhn in *Laboratory Investigation*. Specifically, the appearance of shallow interalveolar septa in alveolar ducts is identical. However, it's not panlobular in distribution; it's very centrally located.

Thurlbeck: Call it alveolar duct emphysema because it's involving alveolar ducts.

Hyde: All right. From here on, I'll call it alveolar duct emphysema. A dog has so many orders of respiratory bronchioles, 3 to 4. If a dog had terminal bronchiole and no respiratory bronchiole, similar to the rat, the emphysematous lesions would indeed be moved much more proximally. The other lesion was a definite increase in the number of interalveolar pores. At higher magnification, some of the pores tend to have strands of tissue in their midst, which were by transmission microscopy proven to be composed primarily of collagen fibers and occasionally accompanied by a capillary.

Stephens: Is the increased size of the pores common throughout the lungs? I recently read a paper about a similar type lesion which was described in monkeys. The control animals had that type of a lesion but it was less prevalent than in the exposed animals. This one happened to be Dr. Bils reporting on the monkey. Also, Jan Nowell, using ozone-exposed rats, saw similar lesions. I forgot what Dr. Bils was doing at that time.

Hyde: He was doing nitrogen dioxide in squirrel monkeys. But to go back, there's a general increase in the number of interalveolar pores and occasionally an increase in the size. If we move up into the first order respiratory bronchiole, some of the alveolar duct outpocketings have a similar type of conformation of an increase in the size of interalveolar pores. In this case, there are some strands of tissue that are collagen fibers primarily, coursing through the airway.

Lewis: Are you giving examples, or are these different types of treatments?

Hyde: I'm trying to give a visual representation of the two types of emphysematous lesions observed. This type of fenestrative enlargement was seen primarily in the $NO_L + NO_{2H}$ group, but also in the SO_x group and infrequently in the $I + SO_x$ and $NO_H + NO_{2L}$ groups.

Lewis: The distal airspace enlargement was seen only in the SO_x?

Hyde: No, distal airspace enlargement was seen in all groups of dogs. I said that the emphysema observed in the SO_x group was the most common form.

Albert: Do you see that Swiss cheese effect only where you see the alveolar duct emphysema, or what you call centrilobular emphysema?

Hyde: The alveolar duct emphysema? Yes. Later, when we did quantitate the lungs that showed increases in interalveolar pores and enlargement, they proved to be the most severely enlarged lungs. In a lung from a raw exhaust-treated animal, you can see a substantial increase in the number of interalveolar pores. This would be a grade 2 where there are many small interalveolar pores, and yet looking at this alveolar duct, there is no enlargement at all. Hence, the semiquantitative grading of interalveolar pores did not reveal a causal relationship between interalveolar pores and emphysema.

Dungworth: Well, that could be restated by saying that there were emphysematous lesions in the absence of any noteworthy increase in pores. There were also increases in pores without appreciable emphysema. Then the most severe emphysema was when the two occurred together.

Hyde: I agree.

Albert: What do those holes actually represent? Disappearance of single cells?

Hyde: No, they're communications between adjacent alveoli through an interalveolar septum.

Albert: Would they be the general size of a single cell or several cells in the alveolar wall?

Hyde: I'm not sure if anyone knows what an interalveolar pore is other than a communicating channel between one alveolus and another. It's lined by type I epithelium. Sometimes alveolar macrophages or type II cells are associated with it. But other than that, I don't think there's much else known.

Question: The question was, is it the same size? If you outline a cell there, it seems to be much bigger than a pore.

Hyde: Sometimes they're larger. They generally tend to be the size of a single cell though.

Kleinerman: Didn't Boatman and one of his associates in Washington do some transmission scope work on these pores and point out that they were lined by epithelium?

Hyde: That's what I just stated. The pores are lined by type I epithelium. They're normal structures. When you see an increase in interalveolar pores, you wonder about their significance. That's why we tried to semiquantitatively rate them and get a feeling for them. Boatman can't make any statement about them either.

Rouser: When you say increase, do you mean increased number or increased size?

Hyde: According to the grading table, the most significant increase would be an increase in size, and a secondary increase would be an increase in number.

Dungworth: They both tend to go together.

Hyde: I don't want to speculate on that point because we have not looked at the ultrastructure quantitatively and before I could make a conclusive statement, I'd like to look at the basement membrane and see if it was retracted or absent in those regions where numerous interalveolar pores are observed. In these dog lungs, the basement membrane looks normal in all respects, as do the type I cells and intercellular junctions.

Bhatnagar: What happens in collagenase or elastase emphysema? Could you describe that?

Hyde: The fenestrations are characteristics of more severe forms of human emphysema.

Kleinerman: Yes. You see fenestrations in human emphysema, but you see them in collagenase as well or elastase emphysema. One of the things that has been passed down, is that if you look at the lungs of infants you do not see alveolar pores. I don't know if this is true.

Hyde: It's the same with dog lungs, although it doesn't come through in Boatman's study. You look at a young dog's lungs, and they have virtually no interalveolar pores, but at 2 years of age interalveolar pores are very prevalent. Similarly, there are many more interalveolar pores in old dogs' lungs that are 13 or 14 years old.

Dungworth: We're comparing control 9-year-old dogs with exposed 9-year-olds.

Hyde: Yes, they're controls compared to exposed, so I believe it's a valid comparison.

Hueter: Some people are theorizing that emphysema animal models must be based on the number of pores the animal has relative to the number a man has. You were using the rate of mouse or hamster, which is inappropriate, when you could use something like the monkey or horse and now apparently the dog. What is your opinion on that?

Hyde: I don't want to take the time to make a statement on that point now. In Figure 3, the surface-to-volume ratio, or surface density, is measured on the Y-axis and the groups are plotted according to increasing severity of loss of interalveolar tissue on the X-axis. Method 1 is the method of using chord lengths; method 2 uses interalveolar septa or number of intersections divided by the total length of the test lines. You can see they are very comparable in the controls, but when you get into the severely affected, the standard deviations tend to vary more with one method than with the other. The distribution of chord lengths shows the greater variance, because it takes approximately 14 samples per field to reduce the variance.

Busch: You mean those limits are ± standard deviation — the brackets shown on the grids? Not a standard error? You don't divide by the square root of the number of samples? I'm just trying to establish what the brackets represent. Do they represent a standard deviation or a standard error?

Hyde: I believe they represent the standard error.

Comment: The variations would have to be enormous to give you your standard error.

Hyde: That reduces the standard error.

Thurlbeck: You're dealing with reciprocals. How do you actually get the standard error of a reciprocal? Do you use ordinary numbers for that, or do you do something mathematical?

Busch: Standard error can be defined for any kind of strange statistic.

Thurlbeck: I have a bias against this particular measurement. I like to use it the other way around, because it's a linear measurement instead of a reciprocal measurement. Finding means for reciprocals means talking about harmonic means and things like that that I don't understand. If one measures the standard error of a reciprocal, do you just handle that as though it was a linear number?

Hyde: That's how I handled it.

Busch: If you have a large sample size, no matter what the distribution of individual values looks like, the distribution of the mean of the values will be a nearly normal symmetrical curve. You can treat it by conventional t-tests with reasonable accuracy, independent of how the distribution of the individual measurement looks.

Hyde: All of these morphometric parameters on Table 2 were derived by using an area-to-diameter-cubed ratio and directly measuring the two parameters of the airspaces with the image analyzer using an areal detection mode and a perimeter mode. From it, one can gain that in a comparison of the control group to the NO_2-high animals, the most severely affected group, the volumetric density of tissue and alveoli decreased significantly. You'll notice also that down the table the numerical density of alveoli decreased and naturally it would decrease then for the right lung.

Thurlbeck: Are these all corrected to TLC?

Hyde: Yes, they are. They're corrected to TLC for the right lung, not for the total. In this case, this figure was the number for the right lung.

Thurlbeck: I want to know about your correction factors. You can guess at what TLC of the right lung is because you know the lung volume. So you then correct from that lung volume to area and linear dimensions.

Hyde: Yes. The cubed root, or the 2/3 root.

Albert: What is it you're measuring, the four significant places out there? That's tenths of a micron?

Hyde: These are just fractional volumes, and these are microns further down the table. These are numbers per cm^2. Here, the cross-sectional area, the volumetric density of alveolar ducts went up and the mean diameter also increased. So from these data I surmised that one logical interpretation is that alveoli or interalveolar septa were lost within alveolar ducts, increasing their volume and increasing their profile cross-sectional area.

Thurlbeck: Your alveolar number is not necessarily an alveolus. By definition, your alveolus is a closed space and that closed space could be the lumen of a duct. You should really call these things B1, B2 and B3, because they do not necessarily correspond to anatomical structures. They're geometrically designed structures.

Question: You may convert free alveoli by getting rid of their septa into what you call a duct?

Hyde: Yes.

Thurlbeck: That's what really surprised me, because my concept of what happens in this lesion is that the lung, as it were, reverts back to infancy. If you start at infancy, primary saccules form the peripheral and smallest structures within the lung and they are then subdivided by secondary crests to form alveoli and alveolar ducts. I conceive of this lesion as the alveolar ducts expanding and expanding, and the alveoli flattening and flattening. I would assume that during this process the alveolar ducts would simplify themselves so you then end up with much larger single enclosed structures. Intuitively, I would have thought that your simplest structure had to be larger in the emphysematous lung than in the nonemphysematous lung. What you're saying is that we so seldom cut through an alveolar duct in such a way as to completely enclose it so that this doesn't occur.

Hyde: That's correct. There are some problems, of course, looking at two-dimensional specimens and trying to make three-dimensional interpretations, especially in altered tissue because we use the rules for normal tissue, apply them to altered tissue and they may not hold at all.

Busch: How did you select the dogs to be included in these samples?

Hyde: In this case where I compared all of the NO_2-high group with the controls, I took the controls that were the furthest away from the mean of the group so that they were the most variable animals for the group. I used a distal airway surface-to-volume ratio measurement for the selection. I did it on all 12 controls because I wanted to compare, in this case, 6 to 6.

Busch: Why?

Hyde: The amount of time required for the computation was much more extensive than by other methods. I only used this method to look at a particular group. Thus, I used an equal number of controls to compare the six exposed dogs.

Busch: You may be biasing the control means as compared to the others since you took an extreme subset. If the distribution is asymmetrical, then by selecting from the extreme tails, you may be biasing the mean as compared to a more complete sample of the test group. I'm not sure if that's true. If the distribution is asymmetrical, you would introduce a bias by not taking a completely random sample.

Hyde: The next type of lesion seen in Figure 8 in a terminal bronchiole is characterized by discrete or confluent aggregations or micronodular proliferations of nonciliated bronchiolar cells. You can see that occasionally they compromised the contour of the lumen, and partially occluded the bronchiole. In this slide, you can see some migratory cells that are located in the distal region of this airway.

Albert: Could you go back a second? What's in here?

Hyde: That structure? It looks like there's some ciliary loss and some accumulation of mucus.

Albert: What are the ridges then? Is that a shrinkage phenomenon?

Hyde: No, it isn't. When you section these SEM samples, it proves to be a hyperplasia. I re-embedded this particular SEM stub and sectioned it through the middle of this lesion to see if it was truly a hyperplasia. It stands up from the epithelium in certain regions such as this example. It is an actual fold of hyperplastic epithelial cells.

Albert: But that's ciliated.

Hyde: Some of it's ciliated and some of it's not.

Albert: And those cabbage-like structures are focal hyperplasia?

Hyde: That's right, and these are more continuous folds of hyperplasia.

Albert: But quite different than that ridge-like hyperplasia. Are they the same cells?

Hyde: Yes, I'll cover that. The most common cell type found in the hyperplasia was the nonciliated bronchiolar cell which had much glycogen in it. Here, of course, there isn't any glycogen present, and yet when you look at normal dogs that are killed, and are processed immediately after fixation, then the glycogen is still present. These dogs tended to be stored for 1 or 1½ years before they were processed, and I believe the glycogen was leached out during that period.

Question: How were they fixed?

Hyde: They were fixed by intratracheal perfusion of a paraformaldehyde glutaraldehyde cacodylate buffered fixative at 30 cm water pressure, in a fixative bath.

Albert: Is this one of those cabbage-like structures?

Hyde: Yes, it is. It's identical to it. You can see in the center there's a macrophage and there's also a connective tissue core to it. Generally you have some type of connective tissue core leading up into the hyperplasia. When they are larger, there is a greater amount of connective tissue found in it, and the basal lamina, of course, goes right along with it.

Albert: What was the exposure of this animal?

Hyde: This particular dog lung? These were most commonly found in the R and the R + SO_x. I'll show you on my grading scheme at the end of the talk, the method of evaluation, and the results. This is to show that if you take a normal dog and you kill it, those cells do have quite a bit of glycogen. Some of these hyperplasias, besides containing the nonciliated cells, have quite a few ciliated cells. This represents a linear fold because we sectioned this in approximately 150 microns and it was still straight up like this. Down in the basal region of some of these hyperplasias, we saw some unusual cells that had basal bodies and cilia in the intercellular spaces.

Albert: Why wouldn't you call those papillomas? They look like a papilloma on a stalk.

Thurlbeck: These are linear structures.

Hyde: I call them micronodular proliferations.

Orthoefer: Initially on histology I did see a couple, and I did call them papillomatous-like lesions in the bronchi at that time. And I think they've been described by Auerbach in his smoking dogs. I think historically they're called epithelial hyperplasias.

Hyde: In mice exposed to 1 or 2 ppm ozone for a long period of time, these were the only lesions that didn't regress over a 120-day recovery period, thus it's not surprising to see them in animals that have been allowed to recover from the exposure. In mice they were the Clara cell population. This cell type that we're seeing that has glycogen in it is the counterpart of the Clara cell in the mouse.

Albert: Did they show any evidence of a high rate of cell proliferation?

Hyde: No, they didn't. There were no high mitotic indices at all. There was no sign of an abnormal cell population.

Figure 11 shows that some of the cells near the basal lamina that have ciliary basal bodies, have cilia projecting from them. I'm sure that's where the cilia in the intercellular spaces originated from. You can see they're fairly close to the basal lamina and at least 5 cells underneath the surface.

In the trachea and bronchi, there were two major exposure-related lesions observed, and these were ciliary loss, with and without squamous metaplasia, as seen in Figures 13 and 14. Here I call it a proliferation because you can't tell whether it's a squamous metaplasia or not. There is ciliary loss along here and it is a proliferative type of lesion. You can see it extends in a papillomatous-type fashion out over the epithelium. There's also a fair area of ciliary loss around it. You can see some cilia over here. This is how you would determine whether there was ciliary loss or not. There are just no cilia there, and here there are.

Albert: At what level on the bronchial tree was that?

Hyde: The level? They were seen primarily in the right primary bronchus. They were seen in the dorsal and ventral regions of the trachea and in some of the groups — specifically NO_L + NO_{2H}, I + SO_x, and SO_x — they were found in the intrapulmonary bronchi.

Albert: Was it any more frequent at the bifurcation?

Hyde: No, in fact, if anything, it was less frequent. I wasn't able to attribute the frequency to any particular structural conformation of the airway.

Dungworth: Could we ask Drs. Thurlbeck, Kleinerman, and Nettesheim to take 10 or 15 minutes to present their summaries on the pathology of the study, and then throw it open to general discussion from there.

Thurlbeck: I must echo some points that Dr. Brain made this morning. This is really a classic and important study, and it's a pleasure and privilege to be associated with it. I can see, just as Dr. Brain can, that in 2 or 3 years time this is going to be a standard reference. For the first time long-term, low-exposure experiments have shown unequivocally lesions in the lung accompanied by functional alterations. If one or the other had been absent, we'd have had a little problem accepting the idea, but speaking as a morphologist, I have no doubt in my own mind accepting the morphologic changes which have been done in an outstanding way and it is a pleasure to see.

I would like to comment about two things that have been described morphologically, the airway lesions, the parenchymal lesions, and then make some comment about the relevance of these lesions to human disease. I was initially disturbed that there appeared to be a discrepancy between two observers essentially looking at the same material. The SRI group wasn't very impressed by the lesions they found in airways but these were clearly described by Dr. Hyde. I think this is a question of sampling and the amount of material available. This is a very good justification for having a scanning microscope because I suspect if one didn't have a scanning microscope to visualize large areas of the lung with high resolution, these lesions would be extremely hard to identify. This has raised the question of being able to use a different instrument to sample properly rather than using random samples of a thin slice of tissue. I'm really quite happy in my own mind that these are real lesions in the peripheral airways of these animals.

There are a couple of very interesting issues related to this which nobody seems to have commented on. One is that the airway lesions appear to be irreversible both functionally and structurally; indeed, the airway lesions may even progress in terms of function. It's been dogma for a long while that we're looking for "early evidence" of "early airway dysfunction" in humans so that we can detect these changes and if people stop smoking, then the patients will return to normal. Now, we do know from tests that small airway function does in fact often return to normal after cessation of smoking. The lesions that we are observing at low levels of ambient pollution may be more severe than those that occur in early smokers. It is reasonably well documented that experimentally induced peripheral airway lesions do not reverse after removal of the irritant. We thus have to question in our own minds the easy notion that small airway lesions, albeit mild, are always reversible. Clearly the ones we are looking at here are not reversible.

The next question that the airway lesions raised is whether they are related to the abnormalities of function that were described, and one would guess that they probably are only poorly related. The reason for saying this is that it is difficult to believe, using conventional McGill and Harvard School of Public Health dogma, that lesions of this sort in the peripheral airways would produce a very large increase in airway resistance. So presumably, this gives us a reason for wanting to be more interested in the central airways in these animals — to know whether there are lesions there and whether there are perhaps functional abnormalities within the central airways. Perhaps the central airways are increasingly twitchy and one may be looking at functional abnormalities rather than anatomical abnormalities within the airways. These again, I'd like to emphasize, appear to be permanent and progressive. What I'm saying is that I wonder whether the peripheral airway lesions that we are looking at are interesting but perhaps not completely relevant to the disturbance of function that we see, and again I think is a hypothesis that should be tested.

I'm much more interested in the parenchymal lesion and I'm going to refer to it as the parenchymal lesion because it raises the question (a) is it emphysema, and if it is emphysema, what sort of emphysema is it? I'd like to stress the point that Dr. Brain has already made, that the increase in compliance is progressive even after the removal of the irritant. We know that in the elastase emphysema model the morphologic lesion is progressive so I would guess that these two were going along together. Now the reason I call it the parenchymal lesion is that I wonder if this is emphysema or not. It brings up an important concept related to the definition of emphysema as an abnormal enlargement of the gas-exchanging portion of the lung accompanied by destructive change. This definition trips glibly off the tongue until someone says, what do you mean by destruction? We now talk about loss of alveolar wall tissue, or a disorder in the orderly structure of the lung, which is the term I like to use. Now, what we've learned from elastase and from today's presentation is that the enlargement of the airspaces and the increase in the pores of Cohn or the appearance of fenestrae do not go hand in hand. It would appear to me that we should look upon these as two separate processes — enlargement of airspaces and the formation of holes within the alveolar wall. I'm going to raise the issue as to whether the lesions that we looked at and the lesions that were measured are in fact a true structural change or whether they represent the result of preparing the lung. It may be finally irrelevant which of the two it is because as I developed this idea, it occurred to me that even if the lesion doesn't really exist in the living and intact chest, there is a functional abnormality within the lung that results in the observed abnormality.

My interpretation of the change that's occurred is that the volume proportion that is increased most is the core of air within the alveolar ducts and alveolar sacs, central to alveoli, that the actual alveoli themselves have not changed in volume. You make the point that the average alveolar diameter is unchanged in the exposed animals. I suggest that the maximum alveolar diameter has increased and this is an old idea of C.C. Macklin who talked about a cup and saucer. He also talked about that at the upper limits of inflation. His idea was that an alveolus was shaped like a cup on expiration, a saucer on inspiration and most of the expansion occurred in alveolar ducts. Now, it is conceivable that what happens in these lungs is that the connective tissue framework, the scleroproteins, has been altered and the lungs have become hypercompliant. As the lungs overstretch the alveoli flatten, the alveolar duct air increases, and the average interalveolar wall distance increases. This may be due to the disorder of compliance, and the primary abnormality is something funny that happened to the elastic tissue. There may be no morphologic abnormalities at FRC in situ. The analogy to this is the old lung. A very similar thing happens if you take aged human lungs and then inflate these outside of the chest. The argument may be irrelevant. Either there is a morphometric abnormality and the morphometric abnormality occurs because of alteration within the scleroproteins of the lung, or there is a morphometric abnormality in life. In any event there is a disorder. It would be nice to see if these lesions occur in situ in frozen dogs.

The final comment I want to make is related to the significance of these lesions, and the significance relates (a) to human diseases, as Dr. Kleinerman and I see them in the portmortem room and (b) to the significance in terms of environmental health. These lesions resemble a very mild degree of emphysema in humans and we would give them a score of maybe 5 or 10 on a 0-to-100 scale. It is the sort of emphysema that one commonly finds in older people, depending on how closely one looks, seldom associated with symptoms, in fact usually not associated with symptoms. There is probably no exact analogy of the airway lesions in the human and they don't closely resemble the Niewoehner and the Kleinerman lesion but I suppose that's the closest human disease that it mimics. What does this mean in terms of the environment because, basically, the lesions resemble minimal human disease that doesn't cause dysfunction? Is this the price that one is prepared to pay for living in an urban environment? It's not causing disturbance, not causing dysfunction, and perhaps we should put up with it. We are getting into a philosophical area, but I think the important point is the lesion that one sees probably would not cause much dysfunction. However, the real question is, given that lesion, are other irritants additive or synergistic? Would these dogs be far worse off than the clear air dogs smoking cigarettes? That of course is the exact analogy that one's always worrying about in the human.

Stara: Are you saying, Dr. Thurlbeck, that you would describe this pathology as an acceleration of normal degenerative disease of the lung?

Thurlbeck: The parenchymal lesion has a very striking resemblance to the exaggeration of the normal human aging processes. What's described in the normal human aging process is a loss of parenchymal tissue, an increase in alveolar duct air, accompanied by either a decrease or no change in alveolar air. One doesn't normally think in terms of focal alveolar wall distortion and destruction in the normal aging process, at least I don't. However, if one looks at aging lungs, one often finds small areas of obvious panacinar emphysema rather resembling what you have seen here, and I regard that as an exaggeration of the normal aging process because we constantly find that in older people.

Albert: How do you know that the normal aging process isn't exposure to air pollution?

Thurlbeck: When I say normal, the average aging process that one sees in the portmortem population. In non-smokers.

Kleinerman: Of course, you can see the aging process in people who don't live in urban environments.

Thurlbeck: The studies that have been done have been in urban areas.

Orthoefer: Would the biochemical change that we saw (i.e., an enzyme which failed to be turned off) be an aging change? Doesn't this occur in aging animals?

Thurlbeck: I don't know what the enzyme changes are that are described in the lungs of aging animals. Now there is a standard collagen/elastin story which is confusing because the collagen and the elastin in major airways, and of pleura as opposed to the alveolar wall parenchyma, have not been separated in the studies.

Gillespie: Dr. Hyde's graphs were on aging dogs, the ones that you say look like those of aging people, which again were from the beagle colony in Davis and not exposed to urban air and not exposed to smoking.

Hyde: They are a different population.

Thurlbeck: It would be surprising if the lungs didn't age. Dr. Albert's question is — if we had a non-urban population versus an urban population of human lungs, how would those lungs look? We don't know the answer to that. I would guess they would both age, and if I could guess again, I'd guess the urban ones would age more judging from this study.

Kleinerman: You know, batting clean-up to Whitey [Dr. Thurlbeck] is like standing in the chow line behind the Cleveland Browns. There isn't much more to do. I personally think that the participants in this study are to be congratulated. I think this is a landmark study. But, as a matter of fact, it really confirms some observations that have already been made, to a certain extent. It's true that the study was done with concentrations of air pollutants which are lower than those which had been previously used. The major concept that has been elaborated is that there can be a development and a persistence of lesions, probably development is the better word, even after exposure to these pollutant materials has ceased. That's the interesting thing, and this of course we reported in hamsters about 2 years ago at the ATS. After we took our hamsters out of 17 months of NO_2 at 20 ppm, we found that the surface-to-volume ratio increased and we reported it with trepidation because we didn't really know what it meant, and about that time Dr. Kuhn reported that there is progression of the lesions after papain. Now we have the ultimate in the observation indicating that there is a probability of continuous destruction even after removal of the exposure. I have absolutely no problem in buying these lesions; I think the care with which the study has been done precludes any possibility that there is error or malinterpretation.

Albert: What lesions are we talking about?

Kleinerman: We're talking about the lesions of emphysema, enlargement of airspaces, and the enlargement of the surface-to-volume ratio which was described by Dr. Hyde.

Albert: I thought that the analysis by the scanning EM there wouldn't take into account the enlargement of the alveolar duct.

Kleinerman: There are so few ducts that their contribution couldn't make a significant difference to the values.

Albert: Isn't that what you were talking about as the aging effect?

Thurlbeck: The surface-to-volume ratio decreases, and this is in fact a reflection of the distance from one alveolar wall to the next.

Hyde: That's correct.

Question: Was that lesion determined correctly immediately after the end of exposure? My impression was that the time they determined that the lesion was present was 2 years later. How do you know what was happening right at the end of exposure?

Kleinerman: You don't, that's exactly the point.

Question: You made the statement, though, that it continued to develop.

Kleinerman: Assuming from the pulmonary function tests that at one time you did not have a change . . .

Thurlbeck: Dr. Kuhn's and Dr. Kleinerman's model shows progressive increase in interalveolar wall distance. They extrapolate to the functional state of increasing compliance, without this measurement.

Kleinerman: No, we did both. Sacrificed at one time and did pulmonary function and the surface-to-volume ratio. At that time, they let them live on 3 months. Now, there's one thing I didn't understand that Dr. Thurlbeck said and that is that you thought the airway lesions were irreversible. I'm not sure that you indicated that they *were* irreversible.

Thurlbeck: They were present.

Kleinerman: What makes you think that they're irreversible?

Thurlbeck: They're still there.

Kleinerman: I wish you would say so.

Stephens: Well, there's a lot of information to reflect on in this particular study as far as I can see, Dr. Kleinerman. After working with NO_2 for many years, we have had an opportunity to observe these papilloma-type structures in rats that have been exposed for various periods of time at higher levels. When animals are removed from the exposure chamber and permitted to recover, such structures decrease in size. We have not observed them to continue to develop in clean air. Certainly ciliary regeneration takes place, usually in a very few days. There is a reversal of the thickening of the basement lamina and it returns to a fairly normal looking structure in a relatively short period of time. Most of my background is from the examination of rat tissue rather than from the dog. We have done scanning electron microscopy in previous studies. We were not requested to nor did we undertake any scanning electron microscopy on the biopsies from these dogs. We did not, however, observe any papilloma-like structures in the light, or TEM preparations of the biopsies.

I was just mentioning to Dr. Hyde the possibility that the agent that caused the dermatitis on the skin of the animals might have been at least partially responsible for the lung abnormalities. He apparently had observed the same abnormalities in some of the controls.

Hyde: I didn't mean to make it sound as though those types of lesions were present in all controls. We only saw it in one trachea in the dorsal region of one control and a small one in the primary bronchus of another dog lung.

Kleinerman: I don't think there's any need to explain the distribution because it's been seen a number of times. We've seen it in our hamsters, too; there are papillary proliferations. The point I'm raising and the question I want to ask is, why does it have to be permanent or irreversible?

Thurlbeck: The question is, if they are reversible, to what degree are they reversible? They're either nonreversible, progressive or partially reversible, because we're sure they're not totally reversible.

Albert: Nor delayed in appearance, like a tumor.

Thurlbeck: You're right, except that these dogs were abnormal in terms of airway function 2 years previously.

Albert: The airway resistance was present only in the $R + SO_x$ and no other group.

Thurlbeck: Let's argue this. Let's take the notion that these things appear after 2 years. Then this requires that something has disappeared and something else has appeared. Well, okay, it's an intuitive argument. But it seems intuitively a very unlikely event that lesions appeared, and disappeared, and subsequently another lesion has appeared, and therefore these present lesions are unrelated. But here these lesions are totally irreversible. How's that for a dogmatic statement?

Nettesheim: Why don't we just say they're still there?

Kleinerman: The objection to this concept is that it begins to lay the groundwork that the epithelium and the changes in the bronchioles may really be a static phenomenon. Once they're there, they're there. That's totally incorrect, because there are turnovers of epithelium. You can go from Clara to ciliated cells. You can go to hypertrophies to hyperplasias. This can happen with dispatch. And we really don't know how rapidly these changes take place. The epithelial nodule may be a distinctively unique lesion, and certainly it's not distributed very widely throughout these cases, as it wasn't in our lesions or in Dr. Stephens'. The fact is that it's probably not a very significant lesion and the important concept that I think we should be left with is that there is a considerable epithelial turnover in these organs and it doesn't stop because an epithelial nodule occurs. It continues, and there's no reason to think that this will go on, at least there's no evidence of progression in our studies and in other studies, so I don't think it should be thought of as a premalignant lesion.

Thurlbeck: I want to make what I thought is an important point. Dogma claims that there is a lesion in the peripheral airways which is reversible. We say that in goblet cells, metaplasia is a reversible lesion.

Nettesheim: That's something else.

Kleinerman: That's another problem in the dogma that you're talking about. That's exactly the sort of thing that you wonder whether or not you might have to challenge, you know.

Thurlbeck: Exactly. Let me finish my argument. What I'm saying is that dogma says that these lesions are reversible. The evidence that these lesions are reversible is really very small indeed and there should be evidence. Let's just leave it at that.

Dungworth: I would like to ask Dr. Kleinerman, of the alternatives explaining why these lesions are seen at the time the animals are killed, which is your preference?

Kleinerman: Which alternatives are you giving me?

Dungworth: That they are essentially unchanged and may be worse than they were at the time of cessation of exposure or on their way down.

Kleinerman: I think that they're regressing afterward, in the terminal bronchioles.

Dungworth: So this is a long delay, 3 years . . .

Kleinerman: You are at a point where you can extrapolate from acute experiments, and this is exactly from where we must depart. Our basis of information is, what happens after you irritate. Do you get a proliferation or a reactive injury? This regresses after a period of time. That's the kind of information we have now. We're producing a new type of experiment, a chronic experiment about which we have virtually no information concerning epithelial turnover. We do have a few fragments of information. Those studies in which you have labeling experiments done, in chronic experiments, would indicate that the proliferation effect in an animal who has become tolerant is somewhat depressed or decreased. If you give an animal 20 ppm of NO_2 as an acute exposure, you get a turnover of so many cells per 100 cells or per 1000 cells. If you give a 20 ppm exposure after he's been exposed for 60 days or 180 days, and then you measure the turnover, you find that he no longer turns over at the same rate and the turnover process is considerably depressed. I think that our dynamics and our thinking about the epithelium have to come from the acute process, and, to me, the big progress that needs to be made at this conference is that we have to set up a pattern of new disciplines in order to deal with what happens to lung tissue and to airway epithelium after chronic injury, because clearly it's not the same ball game as it was after acute injury.

Brain: If you point us to acute models where there's just been one insult that leads to emphysema, can you think of an instance in which the pathology regresses and the physiology progresses? Isn't that what your hypothesis demands?

Albert: There's confusion here because the evidence of emphysema occurred predominantly with the SO_x and the NO_2, whereas the hyperplasia of the bronchiolar epithelium occurred in response to exhaust exposure, so that the progression in the abnormal pulmonary function was in response to a different set of agents than the increase in the mucosal hyperplasia. It seems very unlikely that the abnormal pulmonary function was due to the mucosal hyperplasia, or had anything to do with it at all.

Kleinerman: I thought we were talking about the epithelium.

Comment: You're saying the parenchyma is getting worse but the airways are getting better during the 2-year period?

Kleinerman: The airways are getting better and the parenchyma is getting worse, and I think they're two different ballgames. That's right.

Gillespie: There is some evidence that the airways may, in fact, be getting worse as well, and that's in the $R+SO_x$ group. Their airway resistance increased following termination of exposure.

Kleinerman: How do you know that's an airway phenomenon and not an elastic recoil phenomenon?

Gillespie: It could be elastic recoil. My opinion would be that in order for elastic recoil to cause changes in airway resistance there would have to be substantial changes in compliance. There were no compliance changes present in the R and $R+SO_x$.

Kleinerman: I think that most of us have been talking about the resistance that we think of in terms of peripheral airway resistance and not the resistance of large airways.

Gillespie: That's right. That's why I think it's probably too simplistic to relate the airway lesions that you were seeing in the periphery with the high resistance. I think there must be other things that happened.

Dungworth: Prejudice put me in the camp with Dr. Thurlbeck, but we recognize that we'll have to find out. I think that if we draw a line over a 3-year time scale and say we're looking at the lesion after it's been regressing, it's a little bit hard for me to take. You're right, and we have to set up the techniques and the protocols that will examine that. You have carcinogenesis interests, Dr. Nettesheim. How would you read this, particularly the nodules in the bronchiolar region? A specific instance I do recall offhand is the one paper by Penha and Werthamer (Arch. Environ. Health 29:282, 1974) on effects of ozone where they exposed mice for 120 days, obtained these micronodular proliferations, let mice recover for 120 days in clean air, and found essentially the same micronodular proliferations at the end of that period. That may have some bearing on this.

Nettesheim: There was no evidence presented that would indicate to me that this nodular lesion has any relationship to any pulmonary function abnormalities. There were no data presented that would indicate to me that that lesion occurs frequently enough, in enough airways, that it indeed could cause any functional change. Once those kinds of data are presented, then I would be more open to that suggestion. I do think that these kinds of epithelial reactions might be important in another way than has been discussed here, namely, if we were to learn more about the proliferative rate in these types of nodules, I think the first question that arises is, why are these nodules there? Are they there because of an increased cell proliferation, or are they there because of an increased survival time of the cells? What is the principal defect in terms of cell kinetics? I think that would be an important thing to establish in order to understand, in terms of cell proliferation, what such a nodule means. Why is it there? If one were to know more about this, namely if indeed at the time of observation, 2 years or whatever after the stimulus has been removed, if this is indeed a consequence of continuous increase in cell proliferation — it doesn't have to be — then a sort of indirect relationship to cancer becomes a possibility. There is a good story going around in carcinogenesis circles which is backed up by a number of observations *in vivo* as well as *in vitro* that tissues with a higher proliferative rate often also have a greater susceptibility to neoplastic transformation or, to put it in other terms, there are certain phases of the cell cycle, namely that of late DNA synthesis, in which cell transformation seems to take place more readily than in others. I certainly from my vantage point would be interested in these kinds of lesions not as preoccurences of cancer — I'm not saying that, I don't think there is any evidence for that — but let's say as a weak spot in a system where epithelial transformation by a neoplastic agent could more readily take place. I wasn't here unfortunately for the first part of the meeting. I would have liked to know something more about the composition of the material that the animals were actually inhaling in terms of analytical chemistry, but I think potentially there are quite a few agents in that auto exhaust, of course, that have transforming activity. I really think that's about all I have to say.

Thurlbeck: Are there reversible lesions within the peripheral areas? This is your field, isn't it, Dr. Stephens?

Stephens: I would like to reflect just a bit on the amount of information that we have gathered over a decade of study on NO_2 in particular but also with ozone, in regard to the proliferative lesion and the possibility of its regression. In the first place, when an animal is exposed, you have essentially a 24-hour injury/destruction phase, followed by 24 hours that we refer to as the reparative/adaptive phase. If you compare NO_2 response to ozone response in the peripheral airways, it's somewhat different. The nonciliated cells in NO_2-exposed animals seem to pile up and stratify to form nodules, and it happens relatively quickly. I have a tendency to attribute this to some of the adaptive changes or some of the metabolic changes that involve the relationship of the cell to the basement lamina. I think that for some reason or other the cells do not differentiate properly or do not leave the tissue in a normal manner. The same thing does not happen in rats exposed to ozone. However, Werthamer's study seems to say that O_3 produces hyperplasia in mice, if I understand him properly. Now, whether we can extrapolate this information to the dog and expect similar changes or not, I don't know.

Albert: Excuse me, I don't understand what you're saying. Are you saying that the hyperplasia occurs in the mouse but not in the rat following exposure to ozone?

Stephens: That's correct (in the terminal bronchiole).

Dungworth: We had the same side-by-side species, rat and mouse, in the same exposure, and the mouse has proliferation and the rat doesn't.

Stephens: And the proliferation rate does go down in rats after it reaches a peak at about 24 hours. By 7 days it is back to almost normal. About the type II cells, that proliferative rate does not reach its peak until 48 hours and then it progresses back down to the normal level. We've done a number of chronic studies with rats exposed to 15 ppm NO_2, until the animals were suffering severely from respiratory insufficiency and we've then taken them out and let them recover. They gained weight. Their respiratory rate goes down and they begin to look healthy again. This cycle has been repeated to produce a more severe emphysematous change. If rats are exposed to 2 ppm NO_2, they will survive their life span without significant nodular formation. The proliferative lesions that we're talking about here become quite prominent in a relatively short period of time when exposure is to 15 ppm NO_2. I agree with you, Dr. Kleinerman, that the nodules regress upon exposure to clean air, but don't forget we're talking about exposure at 15 ppm, not at 1 ppm or less that the dogs received.

Albert: Is this the mouse that develops the spontaneous incidence of pulmonary adenomas?

Stephens: No, I haven't had very much experience with mice. That was work by Werthamer.

Albert: Does it increase the incidence of pulmonary adenomas in the mouse? Maybe it's all part of the same process.

Comment: I think they picked a species that had a pretty low incidence of spontaneous adenoma.

Albert: Does it increase the incidence?

Dungworth: They had one paper some time back in which they reported a couple of adenomas, but it really didn't stand up to the light of day definitely. This was a long-time exposure to ozone at the same, I think it was Swiss white mice.

Dungworth: . . . a couple of animals that had what looked like possible adenomas.

Nettesheim: We found, too, that ozone increased the incidence of pulmonary adenomas in C57 black mice.

Albert: So it may all be part of the same process; namely, the finding of the hyperplasia of the bronchioles does represent the very beginning of a neoplastic process.

Kleinerman: Most of the adenomas in mice that are reported are not bronchiolar adenomas. They're adenomas of type II cells, which are alveolar cells. So I'm not sure that you can compare the two unless you are willing to bridge this gap and say that if it happens in one area, it may happen in the other.

Albert: I was thinking of the tumors that were induced by plutonium in the lungs in studies at Hanford where the tumors were adenomas, and the thinking there was they seemed to arise from bronchiolar epithelium.

Kleinerman: That was not in mice, was it?

Albert: No, dogs. Maybe what is being seen here is just a very small step on the same path. It may be that if the animals were allowed to live longer, some of the dogs might have developed pulmonary adenomas.

Stephens: There's a difference between the data that Dr. Hyde presented and those we were able to acquire from the samples that we had, and I would like to discuss it briefly. This study was well done and certainly I accept the possibility that sampling was a problem although our biopsies were quite large. I would expect, however, that if the lesions were present at 3 years after the biopsies were taken that they were probably there at the time of the biopsy. In future design you would certainly want to include scanning electron microscopy. It's a very powerful tool for the kind of study that was needed. We have used the scanning scope before also but at the time we were requested to become involved in this study I believe there was a crisis situation where the experiment had to be terminated. There wasn't very much planning that went into how we would take the tissue or what we would do with it. It was very rapidly decided that we would take biopsies from animals exposed to NO_2 and the irradiated auto exhaust. I believe a substantial question still remains as to the significance of the total data both because of the level of exposure and the conflicting data obtained from various portions of the study.

Orthoefer: I wonder if more shouldn't be said about the fixation process because I feel this is the one thing that really made it possible to visualize the emphysematous-type lesions with the SEM here, and there was some sort of question placed upon the diagram of the fixation apparatus. I think it's the only way you're really going to see these lesions.

Kleinerman: I don't think that Dr. Thurlbeck's concept is very viable under the circumstances. I think it's a nice thought, but I think it has to explain the peculiar localization and the loss, the decrease in $D_{L_{CO}}$, which I think you wouldn't expect if this were just an expansion lesion, unless you're building another parameter in to say that you also lose capillary bed when you have these expansive hypercompliant lesions. So I think it's an interesting hypothesis, but I don't believe it at all.

Orthoefer: After I left those lungs fixed for 16 or 18 hours, I tied them off at the trachea and did displacement studies with them to get the volume. I think if what was said is that we have an artifact then the whole lung would have expanded and this would have been picked up very easily just by comparing the volumes.

Thurlbeck: We know that's the structure of the lung when it's prepared in this way. We know the alveoli are the same size, we know the lung is increased one-third in volume. So the increase in one-third of the volume is in that core of air in the middle. The question is, is it that way during life? I'm trying to say that this is a trivial question. There's no way of guessing what the lung looks like in the chest during life. The air passages may look very different at the highest part of the lung compared to the bottom end. What I'm saying is that one can predict that on the basis of rotting the connective tissue of the lung and preparing the lungs in this way, this is the sort of lung that you would end up with.

Could we just come back to a previous point for my information? What documentation is there of reversibility of airway lesions in experimental animals? Now, the only information I have is a one-shot experiment of my own with a high dose of NO_2, where you get the severe bronchiolitis obliterans that is entirely reversible. That's a one-shot experiment. The only other information I have concerns a Lamb and Reid experiment in which they induced goblet cell metaplasia, which after 5 weeks didn't reverse. Now, do you have published experiments showing the reversibility of goblet cell metaplasia or other small airway lesions?

Stephens: In details of cellular recovery, for instance, it is quite well accepted now that one of the initial things that happens is that there is a loss of cilia and a loss of ciliated cells as a matter of fact. If the animals are placed in clear air and permitted to recover after NO_2 exposure, the cilia generate very quickly. In fact, if there's a mucous layer that develops over the top of the ciliated cells that have lost their cilia, the cilia will reestablish themselves under this layer of mucus even with continuous exposure.

Kleinerman: But in addition to that work in which you actually can show that there is a proliferative response within the first 24 to 48 hours, we published that in the AEC Symposium in 1970. We did that with 100 parts, with 20 parts, and with a smaller concentration. We plotted it over a period of 0, 24, 48, 72 hours, up to 14 days, and described the differential response in differential areas over the respiratory tree from the alveolus to the trachea.

Comment: Which lesion?

Nettesheim: There are many lesions.

Thurlbeck: SO_2, NO_2, or ozone exposures produce lesions within airways. Now what experiments have been done looking at reversibility of any lesions produced by these or other airway irritants?

Albert: Well, I can tell you that in terms of clearance with cigarette smoking in the miniature donkey, you can keep the animal at a level of severe impairment for many months by repeated exposure on a set pattern. And as soon as you stop, it reverts, and similarly with repeated exposures to sulfuric acid, it can build up a response in terms of slowing of clearance and in a matter of weeks it tapers off. So that the clearance effects come and go.

Comment: Neither of those indicates reversibility of anatomical change. It may be the changes of mucous secretion in the quality and thickness of mucus.

Albert: It could be, sure, but that's not what he asked. In terms of carcinogens, Kushner showed for a variety of agents that the initial response is a severe hyperplasia, a metaplasia of the mucosa, which then reverses. The process goes back to normal for a long period of time, and then it starts up again, and when it does, it goes on to cancer.

Thurlbeck: What were the carcinogens?

Albert: Various polycyclic organic carcinogens, and ionizing radiation.

Dungworth: If we're to bring the discussion closer to home we should limit it to what evidence is there that these micronodular proliferations regress or not. Not that there is damage which is repaired, and so on, because I think that is acceptable and not relevant to those micronodular proliferations. If we explained the effect of the ozone in the rat versus the mouse we would get a different story completely regarding the proliferations of the bronchiolar cells. Now maybe the dog is more like the mouse. We don't know. I think that's the particular lesion that we should address in talking about what models are there, or what evidence is there.

Stephens: In our experience, to respond to that specifically, along with the micronodules is a thickening of the basement membrane, or at least a continuing of the basement membrane, sometimes into these nodules themselves. That reverses quite rapidly after being taken away from the exposure chambers.

Dungworth: This is in rats?

Stephens: That's in rats, that's right. I would say that we haven't made a detailed study on the micronodules themselves, but we have done these experiments where they have been in and out of the chamber for extended periods of time. My very strong opinion would be that the nodules in the rat do regress in the recovery periods, and that's on successive recovery periods. But we haven't, as I said, done a detailed study pointed toward that aspect alone.

Thurlbeck: Is this true for ozone?

Stephens: No, ozone does not create the micronodules nearly as much as NO_2 does, at least in the rat.

Dungworth: Dr. Nettesheim, because of the difference between the rat and the mouse with a response to ozone, have you come across the evidence or suspicion that in mice, bronchiolar cells and alveolar cells too, for that matter, are more likely to proliferate in response to injury than those of rats or other common laboratory animals, evidence that the mouse is more prone to develop proliferative lesions in response to irritants, whether it's NO_2 or other irritants.... This is, I realize, a very general question.

Nettesheim: I don't have any comparative data with mice. We have done all our studies in rats and hamsters with respect to NO_2 and formaldehyde, and there both species are highly susceptible to turning on cell proliferation with things like nitrogen dioxide, ozone, formaldehyde, and so on, so I don't know of any data.

Dungworth: I'm thinking of these papillary proliferations ...

Nettesheim: No, I don't have anything.

Stephens: The problem here is trying to understand the process of the establishment of the papilloma. Rapid proliferation takes place very shortly after the initial exposure; however, the cells continue to build up, not because of an increased cell turnover because that returns to normal after a few days. I believe that the relationship established between the cells and the basement membrane gives rise to the papilloma. When continuously exposed to NO_2, nonciliated cells do not differentiate properly into ciliated cells but accumulate and remain attached to the basement lamina, giving rise to the papillomas.

Albert: Can I change the subject just a minute to get a clarification, at least in my own mind, as to what the peripheral lesion is in these animals? The data show a decreased surface/volume relationship in the alveoli. Was that due to a breakdown of alveolar septal walls, or was this the same spotty process that was associated with the enlargement of the alveolar ducts?

Hyde: It was a focal lesion.

Albert: The whole thing is focal?

Hyde: Yes.

Albert: The increased numbers of pores are not?

Hyde: Not the way I graded it, no. I graded it on the entire specimen.

Dungworth: When the increase in pores was seen, was it fairly well distributed throughout the alveolar parenchyma as opposed to being also focal around the alveolar duct?

Hyde: Usually, when I graded the entire stub, I was looking for the entire distribution of interalveolar pores. There was the occasion where the enlargement was accompanied by an increase in pores and fenestrations.

Albert: Therefore, the decreased surface volume effect that you demonstrated was really part of the enlargement of the alveolar duct process, so it was a patchy affair.

Hyde: Yes.

Thurlbeck: The answer is that it's probably both. There are a lot of things going on in these lungs. The volume-to-surface ratio increases as the surface-to-volume ratio, its reciprocal, decreases. And that will result automatically if the lung enlarges. It's just got to. But the point is that the volume-to-surface ratio actually increases more than the volume increases. The volume increases one-third. The linear measurement should change to the cube root of 1.33, which is going to be a small change. In actual fact, the linear change is about the same change as the volume change, so something else is happening besides just isometric stretching of everything. We know that the internal geometry of the lung is being altered. The analogy is exactly the same as the aging human lung. Because you change the internal geometry of the lung, you change the linear dimensions more than you would anticipate. But the thing that Dr. Hyde hasn't told you, but he told me as we were talking, is that in some of these experiments, the actual absolute surface area decreases. Now the absolute surface area can alter, again, for two reasons. One is loss of tissue, and two, the geometry can be altered. One would guess that both of these are going on at the same time at least in some animals. Also we know the proportion of tissue decreases from 11% to about 7%. That change can't be accounted for by just dilatation. I think you could certainly do the calculation, but I don't think you would get a 50% change. You would get a 20 or 30% change; thus there *is* loss of alveolar wall.

Albert: If there is an increase in the volume of the lungs by one-third due to the process that you described, that doesn't sound like a patchy affair, does it? It sounds as if it must happen in a substantial portion of the alveolar ducts. Does that fit the observations?

Thurlbeck: That's a good point.

Tyler: It fits the morphometric analysis quite well actually, doesn't it?

Hyde: The alveolar ducts. As far as the number involved or percent involved, there is a much higher number in the NO_2-high group than in the other groups. As far as the location of the dilatation in reference to the structure that dilated, it's primarily located in the proximal alveolar ducts.

Albert: Well, what proportion of the alveolar ducts?

Hyde: I didn't quantitate that, but the majority of them are in the NO_2-high group. All I was trying to differentiate between was where the dilatation occurs, not necessarily the percentage of alveolar ducts in which it occurred.

Albert: But it's not such an uncommon lesion.

Hyde: It's a very commonly viewed lesion in those dogs, in the NO_2-high group.

Comment: And functionally you've impaired gas exchange. The diffusion capacities are low.

Hyde: The diffusion capacities are down in that group. What would be interesting would be to take individual animals and compare the data across to see how accurately the morphology fits the pulmonary function, but we haven't done that.

Thurlbeck: We're playing word games. I would like to re-emphasize that this model to me looks like my concept of what happens in older people. My idea is that nearly all older lungs on which one makes morphometric measurements have consistent changes that I detailed before. In a minority of older people, one finds localized areas where the lesion is worse, that there's flattening of ducts, there's "panacinar" emphysema. This is what I envisage has happened in your animals, that you have a change that involves most of the lung, but in some parts of the lung is worse than in others. There are two aspects — in each acinus, most of the alveolar ducts are involved at a particular point, but in some acini, some lobules, it is worse than in others. And that's my concept of what happens in some older people.

Comment: Well, let us not forget, it is treatment-related.

Thurlbeck: Oh, yes.

Stephens: There's no doubt that it is a result of the treatment. It's very logical when you see the early lesion. The early lesion is at the level of terminal bronchiole and proximal alveoli. It's not patchy. Every terminal bronchiole shows the same kind of response in the first 3 or 4 days, so if there is any relationship between the first few days of exposure and the end result, we would suspect it to be at this location.

Comment: That's at high levels.

Stephens: That's right at various levels.

Dungworth: It begins to get very spotty down at the 0.2 level, but that's another story. We would like to know why that occurs with ozone, what leads to it.

Albert: One of the things which is naturally of interest is dose-response in terms of building up information related to levels of risk at different levels of exposure. How would you characterize the difference in response, say to the NO_2-high and the NO_2-low with respect to the damage to the lung? Can it be quantitated, say, for example, by the surface-area-to-volume ratio?

Comment: Can it really in this particular experiment? Because you've got another component to this system, too, that is the opposite: the high levels of NO that accompany the low NO_2. It's a two-component system, NO and NO_2. One is high and the other is low.

Albert: I know, but is there any evidence that it does produce lung damage?

Lewis: From our pulmonary function data, the only thing we noted was an apparent elevation of RV. We didn't see the diffusion alterations that we saw with NO_2-high.

Albert: Does NO alone cause lung damage?

Comment: You don't have NO alone.

Albert: No, I mean from other studies.

Comment: I've been out of air pollution for 6 years, but when I first came in here, it was very innocuous, NO.

Comment: NO doesn't really exist very often alone. It's difficult to have it by itself.

Comment: So is NO_2.

Comment: I think people are getting interested in it again and want to look at it.

Stephens: Well, for some time it was thought that N_2O_5 was more toxic than NO_2, bu eventually that was worked out. If you went back and looked at Stokinger's early review papers. His several reviews have been excellent in trying to straighten out all of the data and present that which is meaningful. N_2O_5 was thought to be the culprit, and NO, I think, has always been assumed to be innocuous.

Albert: Well, if you assume it's innocuous, then you have the basis of making a dose-response relationship in terms of surface-to-volume relationships.

Albert: You have the first quantitative dose-response curve for chronic lung damage. Off we go!

Comment: That's a weak two-point curve, but it goes in the right direction.

Stara: I'm interested if you could expand on your comments, Dr. Nettesheim and Dr. Stephens. Did you suggest that you do not believe that there are lesions?

Nettesheim: No, I never said that.

Stara: Or that the lesions were related to the treatment.

Nettesheim: No, what I was saying, is that there's no evidence that those nodules are causing any changes in pulmonary function, because I don't think there are any data that indicate how many of the peripheral airways show such nodules.

Comment: It wasn't measured.

Nettesheim: Right. We just don't know. Until you can say that 80% of all the peripheral airways shows at least one of those nodules, you can't relate the change in pulmonary function studies to the morphology. That's all I was saying.

Stara: Dr. Stephens, these lesions were transient, weren't they? These dogs have been out of the exposure atmospheres for 3 years. They were breathing air that shouldn't have any re-irritating effects and yet these lesions were present in greater quantities in the exposed dogs than in the controls according to all the measurements made.

Stephens: Well, I think it's obvious that there's considerable difference in species and their response. I think the mouse is different from the rat and the rat is different from the dog and maybe the mouse and dog are closer together. I don't think there are enough data yet on rats, for instance. We've never made it a focus of a study to say that the tissue recoveries and the nodules eventually disappear. Have you presented those data, before?

Kleinerman: Which data?

Stephens: The data about whether these proliferative-type structures or nodules do disappear. Is there a reversion in their prominence on recovery?

Kleinerman: Not these nodules. I haven't studied that.

Nettesheim: I thought you said that, in hamsters following NO_2 exposure and recovery, the nodules were positioning.

Kleinerman: Yes, that's right, some of them were still there. But we didn't quantitate the number between the two.

Dungworth: In regard to the correlation between physiologic and morphologic studies, I would like Dr. Gillespie to comment. It seems from the morphologic side, when he said the physiologic parameters indicated an emphysematous-type of change, that his ranking correlated beautifully with Dr. Hyde's morphometric data, and when he listed the indication of whether it was parenchymal damage, that he brought up to the top a mixture of those in which there was the emphysematous lesion and those in which there was the most severe proliferation of the small airway epithelium. You want to comment on that, Dr. Gillespie?

Gillespie: What you said is precisely so. Lung compliance is a measure mostly of the elastic recoil of exchange area. The compliance was increased significantly in the NO_2-high, $I+SO_x$ and in the SO_x. Now, Dr. Brain pointed out this morning that if you had severe loss of communication with some of your parenchyma, then you could have changes in compliance. That wasn't so in any of the dogs. So I think it's reasonably safe to assume that the change in compliance, the increase in compliance, probably relates to a decrease in elastic recoil. I think the most reasonable explanation for this function change is loss of recoil tissue. In addition, those same groups — NO_2-high, SO_x, and $I+SO_x$ — had the greatest change in lung volume. Again, the capacitor of the lung is the exchange area. I believe that there was either a loss of elastic tissue or frank loss of parenchymal tissue. I don't think it can be explained solely on a reshaping of the terminal airways because there was too great a change in volume.

Dungworth: That correlates well with the morphometric data.

Gillespie: Yes, that's exactly right. The animals that had increasing, or at 2 years had the highest, resistance were those that breathed $R+SO_x$. Group I had a high resistance but not statistically different from the controls. These two groups had the greatest amount of morphological evidence of damage in the airways. SO_x and NO_2 groups had the greatest disparity in frequency-dependent compliance, which is another way of looking at the time constants of the lung. The high values of these groups, at low frequencies, is because there is such a high compliance.

When one measures resistance with the oscillatory technique at FRC, the resistance value is related to the large airways. This measurement includes the resistance of small airways, but the predominant influence is the large airways. Dr. Hyde perhaps overstated the case a bit when he said that that doesn't measure small airways. That's not quite true, but you have to have substantial change in the small airways before you see great changes in resistance values.

Albert: Is it legitimate to think in terms of the possibility that the effect of exhaust is to ameliorate the effect of either SO_x or the NO_2? Because, in terms of the surface-to-volume ratios, the SO_x and the NO_2-high are at the far end of the spectrum and the irradiated and the raw are at the other end, and when you combine them you either get an effect in the middle or it's even less than the exhaust itself.

Thurlbeck: The pulmonary function corroborates that, too, doesn't it?

Gillespie: Yes, that's right.

Dungworth: That was the point that I was going to raise next. There are some slightly mystifying aspects of looking at the various mixtures, and one being that with the irradiated exhaust or I + SO_x, there is more NO_2 for instance than in the NO_2 and NO atmospheres alone, but yet the lesion deep in the parenchyma was less. Could this be a particle factor? We were talking this morning about the effects of particles and deposition relative to H_2SO_4. Is it possible that I and I + SO_x lie in the middle, but if you added up all the possible components, they should be worse, that this was a particulate absorption phenomenon that they weren't carried down as far?

Lewis: It's a possibility, but the NO_2 could have been oxidized to nitrate before it reached the actual alveolar zone or even the bronchial zone.

Rouser: I would like to point out the possibility that if the hydrocarbons actually enter that membrane space and compete with things like NO_2 and so on, they could cause a lot less lipid damage. These are physical properties of these molecules.

Busch: I don't know what process you're in, whether you're drawing conclusions which start to be supported by the data, or whether you're building hypotheses. You seem to be building hypotheses. If you are building hypotheses, then it's important not to confuse absence of statistical significance with absence of effect. If you take the difference between a test amine and a clear air amine the measured value may be here. When you allow for the possible limit of experimental error, that's a process of putting confidence limits on the results. If the confidence limits are to abide for the true mean, then the lower limit on the difference doesn't go below difference, so you would say you have a statistically significant difference. Now suppose this difference is statistically significant, and then you have another test group minus clean air where the experimental value falls there and the limit of air falls here. In that case, there would be no statistically significant difference. But it does not follow that this first treatment is different than the second treatment. They both may be showing the same effect, but because of the random variations, this one fell a little bit too close to zero to be called statistically significant. The other one happened to fall a little higher, so you were able to pinpoint it as an effect, but just because SO_x and NO_2-high were the only ones to show significant effects does not mean they were the only treatments to have an effect.

Comment: They showed the most demonstrable effects.

Nettesheim: I think the point he is making is an extremely valid one, because we were indeed jumping the gun trying to figure out why one group shows this effect and the other group exposed to the mixture seems to go in the opposite direction. I think that's the point you're making, that the difference may not be real because of the absence of statistical significance.

Busch: The point I'm making is that if you have an interesting hypothesis, your data may be consistent with that hypothesis even though one of the treatments shows an effect and the other one apparently does not, considered by itself.

Comment: Don't let the data get in the way!

Stara: I think that Ken Busch has in one sentence described our problems.

Busch: We looked at rather simple hypotheses. We compared treatments with means and treatments with other treatments, but we didn't develop every possible hypothesis that seem to be now of interest to this group. It may be that the data are consistent with some of these hypotheses, but, remember, these were not selected to be tested.

Bhatnagar: This is one of the possibilities, and I tried to bring that out earlier today. The difference between SO_x alone and SO_x with irradiated exhaust, it's possibly just simply chemical. The main component of pure irradiated exhaust, the toxic component, is possibly ozone which leads to the formation of the superoxide radical which is supposed to be the most important component of photochemical smog in terms of interaction as an oxidant with biological systems. Now, when you have sulfur dioxide alone, you get bisulfite ion, and bisulfite ion eventually ends up as sulfate. The process continues with the generation to superoxide. So if you have sulfur dioxide alone, you actually also produce superoxide. But in the presence of ozone, the superoxide may not need to be generated in going from bisulfite to sulfate, and the sulfur dioxide may actually compete for the superoxide radical formed by the interaction of ozone with tissues. Because of this both sources of superoxide arise in the irradiated exhaust. Superoxide may be generated by sulfur dioxide alone or be dissipated by its subsequent reactions. So you have at least one component of the toxicity missing here simply because of the competition between these two.

Comment: If that theory were true and tenable, one would suspect that SO_2 would be more toxic than sulfuric acid, and that's not the case.

Bhatnagar: Well, SO_2 is transformed very rapidly.

Comment: No.

Bhatnagar: Anyway, the thing that somebody pointed out there was that bisulfite is very rapidly transformed in the biological system.

Nettesheim: There are other reasons why sulfuric acid is more toxic than SO_2, that have nothing to do with the chemistry.

Bhatnagar: I'm simply pointing out that there's competition for the superoxide ion between sulfur dioxide and the irradiated exhaust.

Comment: You were saying that sulfur dioxide would generate superoxide.

Bhatnagar: By itself, it would generate superoxide.

Comment: We've exposed dogs to 5 ppm of SO_2 for a long time with no impairment.

Bhatnagar: I don't know if sulfur dioxide has any toxic effects on these dogs. The irradiated exhaust does have toxic effects, and sulfur dioxide seems to ameliorate that effect, that particular effect. This is what I've been hearing here.

Comment: That's not sulfur dioxide.

Bhatnagar: SO_x.

Comment: The next generating problem is really sulfuric, which is SO_3 and H_2SO_4.

Bhatnagar: Yes, and SO_3 would compete for the superoxide radical, reduce the concentration of the superoxide radical, and therefore somehow lessen the toxic effect.

Comment: There's gaseous SO_2 in there, too, metered in from tanks.

Bhatnagar: SO_2 would do the same thing.

Stara: I wonder if we could take a moment. Two or three times during the meeting, we have had some questions or problems with some of the statistical treatment of the data, and I wonder if you would, for the record, Dr. Busch, summarize the issue and what you think should be done.

Busch: The serious problem I saw would be sample sizes on Dr. Hyde's data, the number of tissue samples. There were relatively few animals represented, but large numbers of samples, because 20 or so samples were taken per animal, I believe. There is a high correlation, a high degree of agreement between results on replicate samples from a given animal. Depending on the relative size of the inter-animal and the intra-animal components of variance, the sample size needs to be adjusted. If the inter-animal variability is large compared to the intra-animal, then the sample size should be the number of animals. You must first pre-average the replicate samples on each animal and then consider those as single observations where the number of animals is the sample size. That's the major error that I've found. There was the matter of selecting the extremes as a sample size, the extreme deviates from the mean of 12 as a sample for the tissue examination. I don't really know what property that has.

Hyde: That was just on comparing the NO_2-high and some of the control animals in one method of measurement. In the others I did use all of the animals available.

Tyler: He had two studies, this study used all of the animals and then the one in which he had two tables; just the two animals, six controls and six from one NO group.

Hyde: There were about 14 animals that I didn't look at for the SEM and TEM. That was just a time limitation. I looked at all controls and then to see the greatest amount of variability I just intuitively took those that were — I did not select those that were closest to the means of the group for distal airway margins.

Comment: Your morphometric measurements in comparison included all 71 or 72 animals. From this complete sample, sub-samples were selected for your scanning electron microscopy.

Stara: This was exactly my point. If you use all the animals available, how much difference does it make if you take 20 samples from each animal and pool the groups together or if you individualize?

Busch: It makes a huge difference in the standard error for the mean.

Stara: We must be able to analyze the data accurately, because we have a relatively small sample size; if we examine many lung sections of each group, doesn't it improve the statistical error even though we have small number of individual animals?

Busch: That's the way it is. If you believe that the animals' lungs were identical so that in fact result on the average, then under this model you could examine your data to see if they were consistent with that model, to see if the samples were the same from every dog's lung. If they were, then treat them all as one big sample from a master dog, pool dog.

Nettesheim: It still has to be treated such that it is clear from how many dogs the 40 samples are derived.

Stara: In dealing with the dam and the offspring in teratological studies is the sample size the dam or the newborn?

Question: When you take many samples from one animal and pool a lot of animals, does that tend to decrease or increase the mean? If you take 5 samples from each animal as opposed to 20 samples from each animal, would this tend to decrease your standard error?

Busch: It's a matter of how you assess the variation in the mean. I think you've greatly underestimated the variation in the mean by treating the sample size as a larger number than it should be.

Comment: It also possibly allows a smaller difference to become significant if you have a much larger base of numbers. When N is large, then a much smaller difference is significant.

Busch: There is one final point I have to make. I think what may have happened is that the statistical analyses were canned and computerized. We got into the habit of running analysis of variance, and toward the end analysis of variance may not have been applicable.

Stephens: I would like to make a comment about potential relevance to human experience and human exposure, particularly in conjunction with cigarette smoking and the calculation with NO_2 there. I guess the level of NO_2 that was being used in this particular experiment was relatively low in comparison to other experiments that have been done. It appears to me that some of the options between animal species are not open, one to another. We have never seen stratified squamous metaplasia in rats and yet that seems to occur from Dr. Hyde's experiences with NO_2 in dogs and certainly from our previous study with ozone in dogs. Apparently the cells in rats do not have that option open to them. Look at what the human experience might be from the data that must be available. You who work with humans might be able to determine the level of NO_2 in cigarette smoke. A puff of smoke might contain as high as 200 ppm. I guess that has been adjusted one way or another and is down in the order of possibly 25 ppm at the present time. If there is any reality to the amount of NO_2 in cigarette smoke, then the lesions that we see in the dog at these low ppm in comparison must be available or could be appraised from autopsies from human experience from heavy smoking. I'm not saying that the data are readily available but it's something we ought to try and acquire additional data on.

Albert: How can you not test squamous metaplasia in the rat?

Nettesheim: The rat is the animal that more than any other tends to develop squamous metaplasias. There is no animal that I know of that gets squamous metaplasia as easily as the rat.

Stephens: We have never seen squamous metaplasia in the terminal airways of the rat as a result of NO_2 or ozone exposure. If it's in the trachea, that's another question, I have not sampled that often enough.

Dungworth: So you would have to specify precisely where anatomically in the lungs?

Stephens: Right.

Question: Do you have any tissue besides the lung? What tissue did they keep in the necropsy?

Orthoefer: Everything.

Stephens: The material that we have already worked on is still in block form. There may be some tissue from the biopsies but I think that Dr. Freeman still has that.

Orthoefer: You're referring to the biopsy because the rest of the lung is available. All the right side of the lung is still available except for small pieces that have been taken out for pathology. Lobectomy was done on the apical lobe of the left side so the rest of the lung is still available. Scanning electron microscopy could be done with the same size sections and compared with some of the other animals. It seems to me that you got a disproportionately large number of the dogs that had high NO_2.

Stephens: We've got controls plus two exposures, the irradiated automobile exhaust and the NO_2-high which had lobectomies.

Comment: You've got five or six NO_2-high. Five of the eleven were left.

Comment: I don't think it should be limited to the lung. We've gone to a great deal of expense and time to conduct the study. I don't see why not, since they can come up with the money but don't, do detailed histopathology on the brain. We talked about this earlier. There is a theory that there is permanent brain damage at various epidemiological levels. It's never been proven. You've got the opportunity here to look at it, and you've got all the tissues. There are potentially other target organs. How about the blood vessels? CO could have an effect on them.

Comment: Somebody already said they did look at the arteries. There was no atherosclerosis, is that correct?

Comment: Not with any morphometry.

Dungworth: You have to be realistic because each of the studies that we talk about is going to take somebody a long long time.

Comment: First you take some sampling from each group. You've got the tissue and you spent a lot of money to get it. It has never been available before.

Dungworth: All we are saying is that observation is fine. It doesn't show anything that is convincingly different. This means that you are going to have to get into some quantitative sampling and once you talk about sampling quantitatively you're into the whole morphometric analysis. It's a large undertaking.

Comment: With all of these different compounds you are using you should potentially have a whole bunch of different target organs.

Tyler: Morphometric analysis of the brain is tough to do. It's not simple and I'm not sure we even know what tests to use on it. Are we really talking about total neuron disappearance or are we talking about disappearance of buttons? Certainly we see both processes quantitatively happening in certain disease processes or in certain behavioral patterns in some cases other than disease. Both are possible. Doing the stereology there is one hell of a lot tougher than doing it in the lung. It would be a tremendous undertaking. I am impressed by pathologists looking at nervous tissue. We autopsy a lot of monkeys at our shop and these guys came up with this PML lesion which was hard to find and so it was really a sideline with these pathologists. At this point in time, unless there is going to be a tremendous amount of effort expended, I'm willing to take the pathologist's opinion that there were no obvious lesions in the brain. I'm willing to take his opinion that in the tissues that he evaluated, the major vessels were within a normal range for animals of that age. We could measure those till hell freezes over and I don't think we would necessarily come up with anything more.

Stephens: Could you increase your sample size in the statistical problem by at least taking a look at the animals in which the lobectomies were made. It may well be that they were a problem. I know the physiology wasn't done on them. If you use the opposite side from which the lobectomy was taken, it may be a way of improving your data base and satisfying the statistical problem.

Hyde: What he is objecting to is the cumulative calculation of all the animals in samples rather than a per dog data base. It's just a method of calculation, not really the results that much. I don't believe it will shift the results. We will change our calculations accordingly. We didn't have a statistician advising us along these steps so it's natural to make this mistake.

Orthoefer: I feel that throwing the lobectomized in with the normal animals is going to cause a lot of problems because they are not normal. Once an animal has been lobectomized, he has had a lot more insult than with the auto exhaust.

Comment: You don't have to do that. There were some animals in the same groups that were not lobectomized.

Orthoefer: We can't throw them all together.

Comment: We have some control dogs that were lobectomized too so you should be able to see if there is some difference in those controls.

Dungworth: I think the need is not so much for morphometry but to look at the lobes that were taken out with the methods that we have used in the more recent investigation and compare them with pulmonary tissue from 3 years later.

Stara: Drs. Hyde and Tyler both suggested further work with the quantitative pathology of the EM sections.

Stara: I was very much interested to see what the expert here would say about the pathology data and particularly the morphometry because this phase was performed only on the histological sections. Dr. Hyde is suggesting that this work should be expanded into the EM tissue section, right?

Hyde: Yes, I propose the use of low-level transmission electron microscopy, about a primary magnification of 2000, in which you look at the interalveolar septae, examining the component parts, type 1 epithelium, basement membrane and the endothelium. You can count cell populations, measure the capillary density. Just about every measurement that would relate to a significant difference in the pulmonary function.

Stara: The amount of funds requested to be spent is not large. What we need to know is the validity of the work and the reasons for it. There was another phase proposed by Dr. Tyler.

Tyler: Basically we want to take the tissues, go back and look at the bronchi primarily and somewhat at the trachea, using Spicer's histochemical methods which we have applied to tissues fixed in this manner in the past, but have not applied to tissues fixed and stored this long. If the storage were a problem, we would simply use Dr. Orthoefer's blocks from his study which have been in paraffin and hence are not subject to any leaching problem; I don't think that happens that much with the mucopolysaccharides present. Then apply morphometric methods for the evaluation of numbers of units and relative numbers of units of each type.

Albert: Is there anything that can be done to further elucidate the primary lesion in the lungs?

Thurlbeck: Certainly the simplest thing to do is just to look at major and minor airways. It might be useful if you want to do simple things and do things cheap to look at the SO_x group that had the high airway resistance as against the control group. It is easy enough to count and measure dimensions in small airways in paraffin sections. You won't get too much joy out of the major airways, but you can measure all those things pretty easily and quickly. You can measure mucous glands, you can point count, you can do muscle. It's very easy if you're talking of small numbers of animals as a pilot.

Stara: Would it be worthwhile at all after all this discussion of cardiovascular lesions to look at the heart and maybe at arteriosclerotic lesions in the vessels?

Lee: What is the chance of getting Tappel's group to look at fluorescent pigments to examine the acceleration of aging process?

Tyler: This is an extraction procedure; he homogenizes tissue and extracts it, as I recall, and then measures the fluorescent pigments spectrophotometrically. I don't think it can be done on fixed tissue.

Kleinerman: No, it cannot.

Tyler: It can be done on the frozen tissue. I agree this is an elegant method for some tissues but is it really the right one for pigment in lung?

Lee: Rather than the lung, I think the heart samples would be more valuable.

Rouser: Yes, heart and brain.

Tyler: The fluorescent stuff you've seen has got to be lysosomes, doesn't it? He's quantitating in an homogenate the secondary lysosomes which you see in EM.

Stephens: I haven't done that type of cytochemistry for a long time but it seems to me that it is a possibility that it could be looked at. Certainly the numbers of lysosomes in some of those epithelial cells in particular. It might be as valid to do it cytochemically or biochemically if the tissue only permits that.

Lewis: There is evidence of lead toxicity in these studies; therefore, it wouldn't be worth doing. The only question is the biological availability of irradiated and raw exhaust. Is the site of deposition within the bone different? However, it may only be an absorption phenomenon because we are dealing with soluble and insoluble lead.

Hueter: We have only a very poor measurement of lead in the exposure.

Orthoefer: What about the pilot lead study? Don't you think that shows an increase in bone lead of animals exposed to auto exhaust?

Hueter: Yes, but the question is what would it tell us. We couldn't use the information for anything productive.

Nettesheim: I would also say that while there are some interesting things that can still be followed up with the materials still available, it would probably be more productive to use the money and manpower to address the questions that have been caused by this experiment.

Albert: There is just one point that occurred to me and that is you showed some damage to the cilia in the trachea. Was that in the region where the intratracheal tubes were put, and did they have an inflatable cuff?

Hyde: My samples were taken 2 cm cranial to bifurcation of the trachea and that's quite a distance away from the larynx where the cuff would go.

Lewis: All dogs were tubed and it was only found in NO_2-high. It would be very coincidental that in one of eight treatments we could see effects, when they were all handled the same throughout the study.

Albert: Would you always put the cuff in the same place in the trachea?

Orthoefer: Essentially it was placed in accordance with the length of the tube and by palpations along the trachea.

Albert: I wondered if the injury was in the area where the cuff was placed?

Hyde: It would be much lower than that.

Lewis: While we were reporting on it, swabs of the trachea were taken to look for infectious organisms. Essentially everything was negative.

Albert: A cuff like that does produce a lot of damage to the mucosa?

Hyde: I'm saying that where we sampled from was far distal to where the endotracheal cuff was placed.

Stara: We have reviewed the possibilities and we can take it from there. Perhaps a discussion of how this study may help in planning and funding other chronic low level projects would be useful.

13. CRITICAL REVIEW. IMPLICATIONS AND PLANS FOR THE FUTURE

W. Tyler, D. Dungworth and J.F. Stara

This chapter consists of a critical review by a panel of experts of the data presented in the previous chapters. The panel members were: R. Albert (New York University Medical Center), J. Brain (Harvard School of Public Health), G. Kleinerman (St. Luke's Hospital), J. MacEwen (University of California Aerospace Medical Laboratory), W. Thurlbeck (University of Manitoba Medical School), P. Nettesheim (Energy Research and Development Agency), B. McLees (National Institutes of Health), K. Busch (National Institute of Occupational Safety and Health), N. Littlefield (National Center for Toxicologic Research) and G. Hueter (U.S. Environmental Protection Agency). Hosting this review session were: Dr. Jerry Stara (U.S. Environmental Protection Agency), Dr. Walter Tyler (University of California) and Dr. Donald Dungworth (University of California).

Tyler: Today's sessions will address some longer range aspects of this and future studies. Perhaps we should look to future research plans first, as our discussions about the toxicology of this study and about the implications on air quality will be long and a consensus may not be possible. While the experimental plan of our present study and the limitations of the work that we have done are fresh in our minds, we ought to consider first the next long-range study. From our discussions over the last 2 days, we see that there has been some value to this study and that more long-range studies are needed. Perhaps our panel of experts would address themselves first to the plans for future long-term low-level studies in "higher mammals."

Brain: The most intriguing and powerful aspects of the current study are the morphological and physiological correlates. Another aspect of the morphological/physiological correlations which hasn't been done yet, but might, is more attempts to correlate data from individual animals. I'm sure you both, Drs. Gillespie and Hyde, have the numbers of individual animals. I think it would be useful to look at the correlation coefficients between various pairs of morphometric, morphological, and physiological correlates. For example, if, on an individual animal basis, one plotted the changes in TLC relative to the controls versus alterations in compliance, one could calculate the correlation coefficient, "r". By looking at r^2, one would then have an estimate of what fraction of the variability in altered TLC could be attributable to the changes in compliance or to some particular morphometric measurement. One can think of various pairs of parameters that could be correlated on the basis of individual animals: e.g., changes in TLC compared to reduction in surface-volume ratio, frequency dependence of compliance correlated with some measurement of small airway obstruction. Is it possible to quantify the degree of small airway obstruction? Could one quantify the total cross section of small airways? One could try to correlate the total resistance with large airway changes or cross section. Residual Volume could be correlated with small airway changes or with changes in compliance. One could try to develop specific hypotheses about possible mechanisms and then see whether the correlation coefficient supports the hypothesis. It also seems that it might resolve some other questions. In the current studies, it is hard to interpret dead space, P_{AO_2} and P_{CO_2} values because of the anesthesia problem. However, if one correlated those variables with morphometric variables and it turned out that r^2 was a reasonable number, that might give you more courage to say yes, the reductions in P_{AO_2} or the elevations in P_{CO_2} have some relation to the exposure conditions.

Tyler: I think you have raised a very interesting point. Yesterday we talked about what should be done in the future relative to these animals, and one of the things we did not talk about specifically was what priorities should be given to the individual animal correlation using multiple methods of evaluation. May we have further comments on this aspect?

Nettesheim: Personally, I think that to try to correlate the function and morphometric data on an individual basis would be one of the most exciting things to do and I would give that a very high priority, alongside the completion of some of the morphometric studies as we discussed yesterday.

Thurlbeck: I agree with that. I think we should also try to look at the problem of reversibility since you can work with the lobes from the animals and the lungs that were removed 2 years later from the same animals, and from other dogs in the treatment group. I recognize there will be difficulties, but that appears to be a key.

Littlefield: A correlation should be tested in situations where there were two samplings at different time intervals on a specific animal; that is, the reversibility or progression of a specified parameter or lesion needs to be tested. Progression and regression studies are of interest to the regulatory agencies and are relied on heavily for determination of what regulations and restrictions need to be imposed. In instances with this study all that is needed is to take the different parameters that had two samplings or more at different time intervals. A problem may be encountered with the specific sample sizes.

MacEwen: We had a lot of discussion yesterday on whether these morphological changes either progressed or regressed, and there was really no experimental evidence. I was hoping perhaps that some of the tissues that were collected might be in a usable fashion to be recompared. That is, not the second sampling from the animals that were lobectomized but the lobe that was removed earlier. Is that tissue in any kind of decent condition or was it properly prepared in a way that can be compared to tissues that were taken subsequently, at Davis?

Stephens: I think there may well be some small portions of that original lobe that were fixed in a way that they could be looked at. I do not think it was inflated so it may be quite a difficult task. We will only know by taking a look at what still exists.

Nettesheim: I would like to say something about the reversal of lesions before we go on to something different, because it might save you some trouble. We recently did a "reversibility" study after carcinogen exposure. The term reversal can be dangerous and one can get caught in a trap. We induced rapidly appearing hyperplasias and metaplasias with carcinogens and, under various conditions, these disappeared, so we were tempted to use the term reversal. When we took a closer look, indeed the metaplasias had disappeared, but something else had appeared instead which was much less conspicuous. I think the term reversal implies reversal to normal, rather than entering another phase of the disease.

Kleinerman: The important issue to me is not so much the correlation between animals and not the definition of the emphysema, because I think it is unquestionably there. Rather it is what the mechanism of this lesion is. If we are to investigate the mechanism of the lesion starting with whatever tissues are left over, you have to look at the ultrastructure because that is what you have left. Such a study may not be a quantitative ultrastructural study, although that can add some information. The approach should be to ask a question or make a hypothesis about what has happened to the tissue and then specifically look for the effects. For instance, if you think that there is increased lysosomal activity in all the cells, can you find it? Can you really find this change in the interstitial cells or the fibroblasts? Instead of doing a blind study of quantitating everything that may be present in the remaining tissue and coming up with numbers, address yourself to specific questions about what may have happened and try to provide the answers.

Brain: What bothers me about that approach is that the smoking gun is long gone. Even though the lesion may have been initiated by release of lysosomal enzymes or fibroblasts may have been importantly included, they may have done it years ago. The kind of approach that you have described may make a lot more sense if it could be done on a longitudinal basis.

Kleinerman: We have no proof that the lesions really have stopped. There is no evidence that they have stopped, no evidence as to when these lesions began, if they have stopped or are going to progress further. I think that's a hypothesis that could be explored in addition.

Albert: It seems to me that the major point is that this study demonstrates the usefulness of long-term studies in animals. It points up the need for a systematic long-range program particularly to define dose-response relationships and interactions of important components of atmospheric pollution. I think if this study will help to develop a program of this sort, it will have made a major contribution aside from the enlightenment it provides.

Tyler: I think we need to concentrate on what should be our next approach to these animals. There appear to be three main aspects. One is that individual animal data should be correlated. The second is progression versus reversal of the lesion. The third is studies of mechanisms responsible for observed effects. May we have comments on this.

Brain: It seems inconceivable not to carry out those studies. The additional amount of money needed to gather that additional information is small in comparison to what has already been invested. I would recommend it with great enthusiasm as money very well spent to do those three or four things you've just described.

Tyler: I have not heard much discussion about the Reid index. It was very popular in the late 50's and early 60's. Dr. Kleinerman, would you want to comment on that?

Kleinerman: I will comment on it only in a general way. I'm not sure I agree with Dr. Brain about the philosophy of wanting to study everything available. From the point of view of realism in terms of time and money, you've got to ask specific questions rather than try to cover the universe. You can do the Reid index or point count goblet cells or enough things to fill one man's lifetime if you are to study everything about these tissues. You have to get down to asking a specific question or two that may help in the understanding of the things thought to be crucial. If you think that studying the Reid index is a crucial index, which I do not happen to believe, then do it. The crucial question has to be defined and from it the appropriate experimental approach taken.

Nettesheim: I tried to point that out yesterday, too. I do not believe that it is useful in these studies to try the "hospital approach": admit the patient, send him through the laboratory mill and get any and all data you can possibly get. I do not think this approach has ever made any significant contribution to our knowledge of pathophysiological processes. I agree with what Dr. Kleinerman says. We should focus on what this study has unearthed as *the* major finding and try to pin it down further and go from there in designing the next set of experiments. We need to ask what are the crucial questions that are raised by this study rather than to try to determine which out of an endless number of biochemical or physiological parameters might have been changed by the exposures.

Thurlbeck: The Reid index in beagle dogs may be pointless because the glands are so small and they are in the trachea mostly. After SO_x exposure, the glands get big and distended but not hypertrophied and hyperplastic. I don't know what the best procedure would be for those dogs with a high airway resistance comparing them to the normal. I would probably quantitate everything in sight in the major airways and look particularly at muscle. Goblet cells should be quantitated.

Gillespie: I think we need to look at the surrounding tissue in the large airway to try to account for the correlation.

Orthoefer: We have more information on these glands in larger airways. They will have to be quantitated. I have done it on a grading system and it was nearly significant in certain groups of dogs.

Littlefield: It would appear that what is done with this tissue in addition to what has already been done would be guided by what you plan on doing in the future. If you plan on doing a carcinogenesis study, then hyperplasia might be interesting to look into. I agree with what Paul says, that is, you can't analyze everything, however, the studies you may want to conduct in the future should dictate to a large extent what you concentrate on in the analysis of this study.

MacEwen: I would like to return to one point, the need to take the available data we have on the pulmonary function studies, try to put them into common terminology, ensure that they have been given exactly the same statistical treatment, and then find out whether we can plot a dose-response curve for the exposure group with various levels of NO_x.

Brain: Another critical aspect of this study is this: to what extent are the atmospheres used a good model for air pollution. I know you've got piles of data about the atmospheres, but I still haven't been told whether the atmospheres generated are typical of urban atmospheres. Can we dismiss the trace metals? Can we dismiss the other compounds that weren't analyzed? When we look at a physiological change and correlate it to high NO_2, is there anything else that might be causing the observed changes? Are there people here who can summarize the situation and say "Yes, this really is an accurate model," or "No, it's unusual to the extent that . . ." and so on? Another important question is to what extent is the dog a good model? To what extent is the dog more or less sensitive than man? The pattern of airway branching is more "pine tree" than man and there is also more collateral ventilation. Furthermore, the ability of the upper airways to scrub soluble gases may be superior to man and/or some other animals. The other concern is whether we have adequately defined the dose. We have defined the dose in terms of exposure, not in terms of the actual dose delivered. Each dog's dose is a function of his size, metabolism, and level of activity. I wonder whether these factors have been considered. It is true that different animals breathing the same particles do not always receive the same dose in terms of particles per gram of lung. There are significant species differences. I would be interested in any comments that any of you have as to what extent that the dog is similar to other animals and man.

Albert: I would like to urge a better characterization of the actual dose of sulfuric acid particles given to beagles. Another point is that it might be a good idea to take a systematic look at the vessels to see whether there is arteriosclerosis. It would be a shame to lose that data.

Lee: That was one of the suggestions I was going to make. Look at the aorta biochemically and morphologically.

Orthoefer: There are slides of the aorta; also one-half of it is still available in 10% buffered formalin. The other half of a lengthwise split was given to the biochemists.

Nettesheim: I would like to hear what the rationale is for doing things like this. I don't see what would prompt you to want to look at the aorta.

Albert: What about carbon monoxide?

Question: Does that cause vascular disease?

Brain: That assertion has been made. On the basis of what we have heard here there is nothing to suggest doing it. But the assertions that others have made and the magnitude of the problem may make it worth doing.

Tyler: Mr. MacEwen, I think you have done as much CO work as anybody. You've looked at the effects of CO.

MacEwen: We did not see any evidence of change in the aorta or in the heart, but these exposures of dogs were considerably longer than our exposures and I think they are well worth looking at. There have been a considerable number of comments made by epidemiologists, including John Goldsmith of the State Health Department here in California who has claimed that lower CO levels than the dogs in these experiments received have caused disease.

Nettesheim: I would like to say that if you get into this, the aorta may be the wrong place to start. You might be much better off starting with the smaller vessels, for example, the coronary arteries and other smaller vessels. If you are willing to make a commitment to it, that's fine. It is not something you're going to do just quickly or the side.

Stara: The cardiovascular studies were not a part of the original study design, they were started in 1970. I want to make a point that one-half of these dogs was exposed for 5 years to a level of approx. 100 ppm of CO, the other half was not. I do think that the careful analysis of these data may be useful. Several of the investigators have some of the tissues frozen if we want to use them; one of them is Dr. Rouser.

Thurlbeck: There is important material that we haven't spoken about — the small mammals that were exposed for a year. My understanding is that they were negative experiments, right?

Rouser: Yes, they were negative.

Thurlbeck: Could the material be reviewed since there are new techniques available? One has to think about whether one can do a lifetime exposure in some other species, such as hamsters or gerbils, in a shorter period of time.

Stara: Those tissues are not available; they were discarded a long time ago. There are some data available on small rodents exposed to auto emissions. I don't think that we should start another study to clarify further the rodent data.

Tyler: I am hearing a strong recommendation to EPA to evaluate the vessels in these dogs, both the large vessels and the small vessels.

Thurlbeck: I think the point is that you are not very likely to get any answer that's meaningful by saying this is a simple study that can be done easily by staining a few strips of aorta. This is a very tough problem that has been confounding people in the vascular field for a long time.

Albert: There is a perfectly straightforward question that you can ask here. Is there any atherosclerosis?

Kleinerman: That is not as straightforward a question as you make out. The answer that you are apt to get may be influenced by the nature of the diet. These animals do not have a diet similar to that of the human.

Albert: Suppose there is no difference.

Kleinerman: But there is, and the dietary factors, uncontrollable as they were in this study, are likely to have an effect which may overwhelm the effects of the CO exposure. In any event, we would not be certain after having performed a long, detailed and expensive study how to separate the effect of CO from that produced by diet in the development of vascular lesions.

Tyler: Can we get back to the relative priority of correlation of physiology and morphology on mechanisms of the lesion. Dr. Dungworth, you would probably like to talk on that for a minute.

Dungworth: I agree with the comments that Dr. Kleinerman made. I think further ultrastructural aspects of lungs can be incorporated into the study that Dr. Hyde is planning, and should be, so that he is looking for answers to specific questions.

Hyde: I agree with you, but it is not difficult to collect extra data when you do stereology. You don't always know what is going to happen. I don't mean to quantitate everything and I intend to ask specific questions. What are the structures that possibly will be changing, such as capillary density and capillary surface areas? With CO these are specific questions that should be asked. Lysosomal activity requires a different level of magnification but that easily could be another step. One should ask specific questions, but it is not difficult to quantitate other structures while one is in the process of answering the original question. There must be a balance between what you can measure economically and measuring those structures that you expect to change because you may not have asked the proper questions.

Lee: Yesterday the possibility was mentioned of analyzing for fluorescent pigment in these tissues to examine them for possible changes in the aging process.

Tyler: We'll add to our list of possible tasks fluorescent pigment evaluation. Are you recommending lung tissues, other tissues or both?

Lee: I would pick heart tissue over lung tissue. The order of preference would be heart, brain, and then lung.

Tyler: Other comments from our panel of experts on the fluorescent pigment evaluation?

Albert: On another subject, what about quantitating those holes of early emphysema?

Tyler: One can quantitate the holes. There are a couple of ways of doing it. The usefulness of quantitation is for dose-response data and evaluation of pathogens.

Thurlbeck: Numbers are a lot better than descriptions.

Nettesheim: No, that's not true. I think that's a generalization that I cannot agree with. I think numbers are often better than descriptions, but sometimes they are just a fancy way of saying the same thing. I think you have to ask yourself, what am I going to learn if I know in this group A versus group B versus group C that there are twice as many holes? Maybe that is important in this case. I'm asking another question. What do you really learn beyond and above the fact that you can say qualitatively there are many more holes in this versus the other one? What have you learned if you can say there are 50% more?

Thurlbeck: If there are really more. The sort of experimental pathology, when you look at pictures alone is art criticism. I suppose that there are very good art critics. I believe in numbers. It may be that the subjective description of holes in the alveolar wall is biased. You can't bias a machine.

Bhatnagar: We've got a very great resource here with all this tissue around and we are trying to figure out what to do with it. I think the scientific community at large should be given a shot at it. A lot of people who are not here and do not hear from anybody from this group would like to have some of these tissues and look at various things. Lots of times I've seen write-ups in *Science*, for instance, where studies funded by National Institutes of Health or other agencies have materials available and they invite a proposal from outside to see if anybody would be interested in taking some of the material and handling it. If you follow a procedure like that and put a write-up in *Science* or something, describe what we have and ask who is interested and who would submit proposals, then you can evaluate those proposals and take it from there. They can find their own funding to do it.

Tyler: Can we move on then to the next major topic, which is the plans for further long-term, low-level studies in "higher mammals." I'm not trying to bias this toward primates. Primates are in short supply and should be used in critical studies where they truly are the animal of choice. Dr. Brain has made some studies of distribution of particles in lungs of animals of various sizes and we've all worried about dose to the tissue. Dr. Brain, we would like to hear a little more of your philosophy on that.

Brain: We have looked at particle doses in five different species ranging in size from mouse to dog. We have completed some experiments where we have these five different species breathing the same particles at the same time. If you express the data in terms of particles per gram lung, you do see systematic differences which in part are predictable on the basis of different metabolic rates and different ventilation per gram body weight. It is in the direction of smaller animals getting a higher dose. There may also be some systematic differences in collection efficiency but it depends on the particle size. The main point is to raise the general problem that very frequently we define the dose in terms of exposure rather than in terms of amounts deposited or absorbed in the respiratory tract. There are many other things that can influence dose. We did an experiment where we pre-exposed mice to a cage environment for a half-hour and then added some naive mice and let them both breathe the same radioactive aerosol. The naive ones had 2 to 3 times as much particle deposition in their lungs as the ones that already had been there. During the first half-hour in a new environment the mice were very curious and active. Other factors may also be important, temperature for example. There is a zone of thermal neutrality and if the animals are warmer or cooler than that, they tend to breathe more. Thus temperature can influence the dose to the lung. In this study my question is; do we know whether animals in different groups had the same ventilation? Did auto exhaust or the other agents added affect their level of activity?

Gillespie: I agree with everything that Dr. Brain said and certainly that will have to be taken into account when we select an animal. We're going to have to know what its neutral zone is and what its ventilation is in the environment at any particular time. One of the things that I think came out very clearly is that we need tests, a variety of tests to study individual animals sequentially, knowing what they were before they go in and then watching them as they go along. One of the problems that we have in physiological monitoring of the cardiovascular and pulmonary system is that of size. It is true that you can study in a rather sophisticated way the function of the pulmonary system of the rat, but it's very tedious. You have a huge problem of measuring flow events from the lung of a rat because very soon the equipment becomes the overwhelming resistance in the whole system. As Dr. Brain knows, Dr. Leith and his co-workers labor endlessly trying to measure accurately the flow events from these small mammals. The advantage of having an animal of some size, something like a beagle or larger, is that it allows large-scale monitoring equipment with fewer technical problems. In a practical sense, when we select an animal it is going to have to be one of size.

Stephens: We have had extensive experience with the morphological response of animals exposed to NO_2. This background includes both acute and chronic exposures of a variety of animals over a significant portion of their life span. We have also studied animals permitted to recover after long-term exposure to 2 or 15 ppm NO_2. This experience leads me to the conclusion that care should be exercised before one comes to the conclusion that the changes reported in the conducting airway are due to exposure to NO_2, at such a low level as was used in this experiment.

Dr. Freeman has been exposing a number of monkeys to 2 and 9 ppm NO_2 for up to 10 years. Some of these have been sacrificed and preliminary observations have been made with both the light and electron microscopes. Unfortunately, we have not examined enough animals yet to make any statements at this time. My point is that these animals are being exposed to comparatively high levels of the gas and have survived for substantial periods.

Question: Have you ever run a pulmonary function test?

Stephens: Yes, and it is my understanding that the pulmonary function tests for a single animal do not correlate well from one test to the next. At least that was the case several months ago. These tests are done in Dr. Freeman's laboratory and I am not certain of the current data.

Dungworth: I would like to ask Dr. Brain a question concerning the relationship between an ambient concentration of a pollutant and the dose that the pulmonary parenchyma actually receives. This relationship is going to vary for different species of laboratory animals. I wonder if he has any suggestions as to the one or two species of experimental animals for which the relationship between ambient concentration and dose to pulmonary tissue would most resemble man. Then we should not be introducing another variable in concerning ambient concentrations for human populations and chamber concentrations for experimental animals.

Brain: I don't suggest comparisons to man as the only guide in selecting species for experiments. I think there are many other considerations such as cost. Another problem is that a free-ranging animal breathes with quite a different pattern and gets a different aerosol dose than one who is breathing through a nose cone shoved in a little tiny chamber.

Littlefield: I feel we should discuss the objective of what you want to do next. We are talking very generally at this point about animals. Unless you know the objective of the study and what you want to do next, you really can't get down to specifics.

Nettesheim: I just want to support this statement because I think this generalized discussion might be very helpful. Statements to use for taking ambient air contaminants are of no use unless you can define specifically what it is you are trying to determine. Only then can you decide what the right species is and whether the ambient air level used is the right one.

Gillespie: One objective I really feel we need is to have some spots or more sampling points along the way so that we can extrapolate about time-dependent changes during the course of exposure. I think they need to be functional, morphological, and biochemical.

Tyler: I think we really need some U.S. Environmental Protection Agency (EPA) advice as to what kind of objective they want achieved in future studies.

Stara: We have with us Dr. Albert who has the knowledge and who was during the past year deeply involved in planning and developing objectives for the EPA Office of Research and Development to solve the problems that EPA is facing from the standpoint of required regulatory actions. We invited also representatives from three other federal agencies that may contribute to the question what it is that their organizations need in terms of long-term studies. On that basis we can develop future programs. I don't think that we can just say, let's study NO_x. I would like to ask Dr. Albert if he would give his views on EPA needs.

Albert: The things that are being talked about are: a fine particulate standard, a short-term NO_2 standard, an emission standard for sulfuric acid from automobile catalysts. That's about it, I think. It is difficult to talk about what goes through the minds of people at the regulatory end. The biochemical aspects of the problem are not necessarily the dominant issue. It depends on the protocol considerations.

Tyler: I think what Dr. Albert is saying is that to survive in the higher echelons of the EPA, you have to be a politician and not a biologist.

Albert: Not exactly. There is an essential need for biomedical data. They prefer to know what levels *won't* produce an effect. All they really want to know is what level won't cause any harm. When you tell them that you can't really say, they find that difficult to live with. We know what the ground problems are. We know that there needs to be work done to define the nature of the pollution effects, both qualitative and quantitative. What, in all the mixture these animals have been exposed to, is producing nasty effects and what is the nature of the dose-response relationships? There was a serious question raised yesterday as to whether the lesions observed in dogs are comparable to those seen in humans.

Stara: There are five ambient air pollutants for which a standard was set: carbon monoxide, hydrocarbons, nitrogen oxides, sulfur oxides, and particulates. The reviews of the present standards are to be done every 5 years based on new information that may determine if a revision is needed. There are no ambient standards for a great number of air pollutants; but with the new Toxic Substances Bill, it is obvious that some regulatory action on many of these pollutants will have to be taken. Do we need to study in more depth the regulated ambient air pollutants, or do we need to study the compounds that we know less about? Where is the greatest need? For example, the EPA is facing a court decision regarding 65 compounds in water; more information on all these materials is needed. I was hoping that in the discussion of proposed future studies this group would address some of these issues.

Hueter: I think the people in the research arm of EPA, and I am not sorry to be there, are continually harassed by trying to fight the pollutant-of-the-month approach. Although EPA has been charged to produce documents relative to control of 65 substances in water, they're not going to do that based on any research that's been done, to my knowledge, within the EPA. There is no time to do that.

Albert: That's water.

Hueter: I don't care if it's water or air. If they had 65 air pollutants to address, they wouldn't base that on any research that we were going to do at this point. It would be based on the literature available now. As far as any chronic study that is to be done, people like yourselves and other people in the country and the world should identify now those areas in which a chronic study has a chance for producing meaningful data for application 10 years from now. It might be carcinogens or fine particulates, whatever that means. The EPA has not decided how they are going to approach the problem of respiratory particulates. NO_x is still a major problem because the standard which was set in 1970 was based upon one study that was done in one place, which has been subsequently criticized. It should never have been set.

Stara: Initially, that was also true of CO.

Hueter: We lucked out on CO because subsequent work more or less supported the standard.

Kleinerman: It's obvious that you've got to give us a little more guideline, a little more focus on the objectives. Are we supposed to help in defining experiments that will determine the agent or the combination of agents which are the initiators and in what concentrations? Is it more important to focus on defining experiments which give you something about the mechanisms of action of these agents? Is it also important for us to define safe limits, because to design experiments with regards to safe limits of these agents would be different than talking about a long-term chronic experiment. Do we have the money and time to initiate another 10-year study to answer these questions, or are you more interested in finding a model which will give us some of these answers in a year or two or less if we go to a different kind of species? The kind of answers you are going to get from addressing questions to us will depend in large measure on the guidelines you give us to some of these questions. I would like to reverse the role and ask you, can you answer some of these questions? If you do, I think you will get a more succinct and definitive response from all of us.

Albert: The concern is with public health and the magnitude and nature of the impact. The issue of mechanisms comes into play in giving guidance to the interpretation of the quantitative data. This gets particularly important at low level responses where the actual observed data have so much uncertainty that the way you draw the curve depends on what you think is the underlying process. For example, if you believe that you are dealing with a one-hit curve, you tend to draw a straight line through a scatter of data. Mechanisms are the basis for understanding the nature and the magnitude of the problem, but *per se* don't figure in at the level where the decisions are made about regulatory action. Looking at the SO_x data, from the analysis, you get an effect in the production of emphysema. Maybe the major health effects from a regulatory standpoint are not those on airway resistance but the induction of chronic lung damage.

Gillespie: I think it's impossible to get into the framework unless you say, here we are, here are our skills, and here are our backgrounds. Just as you pointed out, we have come this far and we've shown these things, some of them not as definitive as somebody that's in the regulatory field may wish them to be, but they're still going to be more useful than a lot of other things that you try to do or try to anticipate doing. I think we're just absolutely going to go round and round in circles if we're going to try to do what we think will please EPA. First of all, I think it will be wrong, and second of all, it will very likely be outside the sphere of the best skills that we have. The exciting things that I see coming out of this last study really weren't those that are going to set levels. That may be a side product. But the exciting thing, I think, is that we have shown under these conditions a very interesting dysfunctional and structural lesion associated with reasonably well defined pollutants or irritants, and you can look at that as being a response of the lung, a reaction of the lung, or you can look at it as air pollution, whichever pleases you.

Albert: I would just like to raise the issue of asking what actually has been observed? And I think it's not at all clear. If you look at your data, the biggest effect of course is the NO_2-high, and the next one is the SO_x. But if you look at the irradiated exhaust, the irradiated exhaust has more NO_2 than the NO_2-high. The NO_2-high has an average of 0.64 ppm NO_2; the irradiated exhaust has 0.94 ppm. It's one-third more NO_2. You put the irradiated exhaust together with the SO_x, and you get a smaller response than with the NO_2-high. Now, SO_x produces a big response, NO_2 produces a big response. You take irradiated exhaust, which has more NO_2 than the NO_2-high, and you put it together with the SO_x, and you get a smaller response. The problem is, what is going on?

Kleinerman: That's still the first question we asked. Do we determine which agents or combinations of agents are the initiators, or do we determine mechanism?

Nettesheim: We need to make that decision. I think we should forget about the regulatory agency. You have done the experiment and I think what should come out of this now is to decide what the most important question is that has been raised by this set of experiments. That's what we should agree on. The decisions which the EPA or somebody else finally makes, that's their problem, not ours.

Stephens: The point I wish to make is in conjunction with the design of any future experiment using chronic exposures.

The first large scale chronic exposure study on lung that I am aware of was done in the Los Angeles area over 15 years ago and the resulting data were not very useful. The reason of course, recognized early in the experiment, was that the level of exposure (ambient air) was too low and inconsistent to be accurately interpreted. The data obtained were equivocal, giving rise to speculative conclusions that subsequent investigations have had to reinterpret. In regard to the present investigation, I believe Dr. Albert has a point in suggesting that there are significant questions about the importance of the current findings. He points out the inconsistency in the data based on the level of NO_2 exposures.

In any case, during the past decade there have been a substantial amount of data obtained from animals exposed to somewhat higher levels of various pollutants.

These experiments have included acute and chronic exposures with the pollutant being administered either continuously, or intermittently. With this extensive information to draw on, we are probably now at a point where a useful long-term study can be designed.

The data should be individualized so that the progression of the disease could be assessed more accurately. Two or more levels of exposure would be necessary, one at a level approaching that found in severe air pollution and at least one at a significantly higher level. With our current understanding of the response of tissues to single pollutants, perhaps a simple mixture of gases and particles would be most productive. Animals with both short (2 to 4 years) and long (5 to 20 years) life spans should be included.

In brief, I believe there is ample information now available to construct a major chronic study that would give unequivocal answers to at least some of the major health concerns related to the exposure of lungs to toxic gases and particulates but much time and effort should be devoted to the design because of the expense involved.

Stara: I wonder if Mr. Malanchuk would comment on this. Is it possible that the cycling of the engine may have influenced the emissions to produce variations in the NO_x levels? It certainly does affect it, and I wonder if the data you are talking about might be misleading because of that.

Brain: I think that's an interesting question. It relates to air pollution in general and relates to flexible fuel strategies for power plants specifically. Is it occasional and infrequent peaks that initiate and cause disease, or is it the long-term average level. I think that's an interesting question and, to my knowledge, it hasn't been addressed in any chronic study.

Stara: Intermittent level exposure versus constant level exposure?

Hueter: This isn't a question of intermittent exposure, though. It's a question of do you have to cycle the engines in order to be able to get the pollutants that we're interested in. For instance, NO_2 is generated when the engine is under a certain phase of the cycle, so these might be average concentrations, but they certainly did vary throughout the day.

Brain: It sounds like, at least in the irradiated exhaust, those chambers are large enough to damp out any cyclical changes even though what's coming out of the tailpipe may be going up and down. That should be smoothed out by the time the animals breathe it.

McLees: I would like to know if anybody knows the chemical species involved in any of these mixtures. Has anybody looked at free radical concentrations in an enormously transient species that can be very, very devastating from a physiological point of view? Superoxide is a good case in point. I think it's impossible to work out unless someone has really done very, very detailed physical chemical studies that I know do not exist in these areas. I don't think that apparent inconsistencies between exposures are a difficult thing to explain away, because I think you don't know the chemical species you're working with. I don't think it really makes the data terribly difficult to interpret. It seems to me that you have demonstrated a very important thing. Low levels have caused unequivocal biochemical, pathologic, and physiologic changes in the lungs. To me, that is a very important piece of information.

Lewis: Everyone ought to be put in a real world situation and try to run one of these studies in 5 years, and imagine the cost and prioritizing everything you would like to be able to study. One could spend a lifetime and billions of dollars just characterizing the atmospheres. And on top of that, consider the economics and logistics to encompass the biological phenomena that go with atmospheric characterization, the appropriate species, the appropriate tests. And a very good point that Dr. Gillespie brought up, you don't have every available expertise on hand while the animals are being exposed, and you have to match experts with what you're trying to achieve and all the other primary considerations that are there.

Tyler: Dr. Littlefield, you had a comment?

Littlefield: I'm not sure it's applicable anymore. What I was hearing at one time is that they're actually talking about two basic types of studies here. One is a chronic dose-response study and the other one is a factorial type study where they study individual components and then try to factor out the actual component that is responsible for the particular response.

Lewis: That may have been an oversimplification. There are many complications in exposure situations such as antagonism and synergism. For instance, we know there were a large number of nitrate particles in the chambers with irradiated exhaust which was not true for the NO_x chamber. But it's difficult to simulate the real world situation under laboratory conditions. Animal activity and all these other variables cannot be controlled for 5 years. You have to make certain compromises because of practical limitations in numbers of animals and chambers. Everybody said we should have studied more irradiated auto exhaust; we couldn't generate more irradiated auto exhaust.

Hueter: The same way, if you could control the activity and so forth, how would you apply that to a study situation for a population of people? You wouldn't just apply it to a set level of activity.

Brain: I'm not suggesting that it necessarily be controlled, but it might be possible to measure it. For example, if because the animals felt sick or irritated in one of the chambers they exercised less and breathed half as much, that might be an important part of the discrepancy that Dr. Albert described. Maybe the animals with the auto exhaust don't feel well, are inactive, and they just lie around and breathe only half as much. I doubt that's true, but perhaps it's a possibility. It's important to know in a situation like that whether the ventilation and the dose are similar in all groups. We assume that the dose of NO_2 was defined by the chamber concentrations, and I'm saying that this may not always be the case.

Lewis: As Project Officer through much of the actual exposure part, my clinical interpretation was that there were no differences in activity among chambers, etc. The maximum activity was at night when there was no exposure and they were going to be fed. There would be sporadic activities when we had visitors or something like that. But the greater part of the day, they were either lying around or relatively inactive.

Brain: It sounds like an unlikely possibility, then, from what you say.

Lewis: Yes.

Hueter: We actually at one time, I believe, sent someone through and we were counting respirations on these dogs over a period of weeks and comparing respiratory rate across chambers and, as I recall, we couldn't detect any differences.

MacEwen: I don't think you could ever detect it in a long-term experiment, because it will change from day to day. You could for short-term acute experiments, but not for any kind of chronic experiment. They've got to average out pretty much the same or else it would be obvious in their growth rates and in other clinical measurements.

Orthoefer: As far as what Dr. Lewis was saying, the people who worked in the chamber room and took samples did not excite the dogs, but if a stranger went in there, the dogs all became very excited and barked and climbed all over the chambers. This type of study could be very stable if you keep strangers out and the amount of activity was programmed. The problem is what do you allow visitors to see and not to see. If you hide the study then you are suspected of doing something out of the ordinary. If you let people see the animals then excitement occurs, injuries occur and problems develop. I don't know the answer, but in future studies using larger mammals it will have to be dealt with effectively.

Lewis: Just a sidelight, the maximum excitement occurred if one dog jumped out of the chamber and ran down past all the rest of them while they were still in the chamber - that's when they really got excited. But that was very occasionally.

Thurlbeck: I'd like to follow up Dr. Gillespie's point. We ought to recommend that someone look at mechanisms of disease production. We have a lesion, we know its functional effect, we know its relevance in terms of environmental exposure, but we don't know the mechanism of production of that lesion.

Dungworth: That's the point I would like to make, that if we asked everybody around the room what materials and methods were necessary for even minimal effective experimental design, we could obtain the highest common factor. But even with that, we're talking about a large experiment and one that is going to cost a lot of money and needs a lot of forethought, and probably is going to need interagency funding.

Kleinerman: You mean, decide where to begin to study mechanism? Do you want an expression of whether the study of mechanism is the way to proceed? If we're talking about mechanism then clearly we're back to the problem of how to produce emphysema. That problem revolves around the balance of proteolysis and antiproteolysis. One can get into the way at any point, measuring what is happening in terms of proteolysis in the lung or serum at various times during the period of injury and repair. One of the specific problems that needs to be answered is the question of how chronicity can produce lesions so distinctly different from those which are present in the acute phase.

Nettesheim: What I would like to see us do is to ask everybody present what, in his opinion, are the most important issues that have been raised by the study and that should be proved; not in order to generate an agreement but rather to get an idea of what the various people with their particular expertise conceive of as the most important questions that have been raised. I think the material that would come out of that kind of discussion could provide the basis for formulating ideas and defining priorities for future experiments.

Tyler: We should address in the short time that remains why EPA should be interested in chronic studies. Chronic studies do cost a lot of money. Dr. Brain, will you start please.

Brain: The mechanisms involved and the damage produced by acute exposure may be very, very different than the lesions produced by chronic exposure. Whether the real threat of air pollution is bronchoconstriction or whether it's the kind of progressive, irreversible lesions that have been described is crucial to determine. The concept of a lesion which progresses after the termination of an exposure is crucial. Perhaps one could even suggest that this study was not prolonged enough. Another crucial question we haven't discussed is the whole idea of impairment. To what degree were these dogs really impaired? The degree of emphysema produced when compared to humans is not very serious. The same thing is true in terms of the physiological evidence. The changes were not life-threatening. If you asked the dogs how they felt, they probably would say "fine." An important unresolved question is: would that lesion have progressed to the point where it would cause a drastic impairment? Would it even become life-threatening? That's an unanswered question but I suspect it may. The rate of change over the 2-year periods in some of the groups was severe. RV doubled over a 2-year period and if one extrapolates that for another 4 or 5 years, one might reach levels which are life-threatening.

Kleinerman: The question of why we must pursue chronic studies comes down to two basic reasons. First, the concentrations that have been studied are realistic in terms of the exposures to which urban populations are exposed. Secondly, we are addressing a major public health problem to which we have no answers, in terms of understanding disease processes.

McLees: I would like to turn it around and say, in view of the results that you have presented over the past 2 days, how could you not do chronic studies? You have shown, from the result of a chronic study, using low-level emission, pathologic, physiologic, and biochemical changes in lung. You don't understand them completely. You don't even know what in the mixtures that you've exposed these animals to is responsible for it. It doesn't make complete sense, as

Dr. Albert pointed out. I really turn the question around. I don't see how you can get by without doing more studies. Perhaps they need to be longer and designed somewhat differently. I don't see how you can get around not following this up and getting some idea of what's happening, the evolution of the disease process and what it's like.

Tyler: Thank you. Dr. Nettesheim, why don't we pick you up next, please.

Nettesheim: I will agree with everything that has been said. In addition, I would say yes, do chronic studies. But, there are various ways of doing chronic studies, and I can conceive of pursuing some of the questions that have been raised by the previous study but taking a somewhat different track. I'm not so impressed with the necessity of staying with near-ambient air levels. I would, for example, if I were given the charge to design new experiments, use two or three times the concentrations that have been used attempting to induce similar kinds of lesions perhaps in a matter of 2 years rather than 5 years. Then I would address myself to the question of reversibility of some of these lesions. I think this chronic experiment has raised enough important questions concerning mechanisms as well as concerning which the crucial factors in the mixtures are. These questions should be provided in a somewhat modified experimental design which yields results within a shorter time.

Thurlbeck: I think the only thing I can do is echo Dr. McLees' comment. These experiments show unequivocal evidence of emphysema. To me, it is self-evident that further experiments have to be done. It is the EPA's responsibility and it is also probably the only funding mechanism for long-term exposures. I assume that it is unlikely that other agencies are going to get involved with this sort of work.

MacEwen: Back to the question of why do chronic studies. I think there's no question about the need for chronic studies, for EPA or any other regulatory agency that is going to have to define ambient limits for exposure. My only concern is whether what has come out of this study is adequate for defining ambient limits. I'm not sure we've got enough information yet in dose-response. We have a positive finding and yet we don't have a negative finding. We don't have a lower limit of response. I believe that EPA should take the finding of this study and undertake additional studies designed to give a higher level as well as lower levels to define where the response ends and to try to quantitate the degree of impairment in terms of concentration or dose-response curves. I think that is the more important thing to define, both the NO_2 as well as SO_x, and I don't know how you're going to do it with irradiated exhaust very easily because you can't dilute it very easily without changing your whole exposure pattern in terms of CO and everything else involved in that complex mixture.

Littlefield: Well, to make it unanimous, I agree that the best way to get at this problem is the chronic study. This is the real world. In an evaluation of a literature review, whether it's for carcinogenesis, a pesticide, or for an environmental chemical, most of the studies are invalidated because the exposures are too short. This is in my experience, anyway. If you try to get an effect on an acute or subacute study, it is often necessary to raise the dose to abnormally high levels which may cause a different physiological mechanism to be brought into play and you begin to get a false picture. Therefore, a chronic study is the best way.

Hueter: I also believe in chronic studies, or else I wouldn't have got involved in this thing in the first place. But I just wonder how many other federal agencies *are* doing chronic studies. Is National Center for Toxicologic Research (NCTR) doing chronic studies? I really don't know.

Littlefield: That's essentially the prime mission of NCTR, that is, dose evaluation at the lower end of the dose-response curve, which essentially is a chronic study.

Hueter: Really, EPA is not the only federal agency in a position to fund chronic studies. It might perhaps be more appropriate if some of the federal agencies might consider setting up a special group to do this kind of work. Their research people do all these quick response studies on the lung. Yet, at the same time, experts like this group rightfully tell the agencies they ought to be supporting chronic studies. There's no way they can do both at the same time.

Albert: As I said before, the main contribution of the chronic exposure studies is the demonstration of effects which are quite different and, in my opinion, much more serious than those that are induced by acute studies. Besides which they supplement the epidemiologic studies in a very useful way. Because one thing the epidemiologic studies haven't done is to answer the question, to what extent does air pollution really produce emphysema and chronic bronchitis. This is aside from the issue of whether or not it aggravates pre-existing disease. But I think one of the major gaps is whether air pollution really does play a primary role in causing irreversible destruction of the lung.

Thurlbeck: One additional reason for chronic studies is that other than accident, suicide, and homicide, all the important causes of death in man are chronic diseases.

Busch: I don't think it's important to simulate community atmospheres. I think it's more important to get information about particular components of the exhausts which we breathe which we can identify with health problems. Some components are being given attention in the EPA regulations; SO_x is one, with respect to catalytic converters. If we confound SO_x with the whole exhaust mix, we haven't perhaps gained as much information which would assist us in making decisions about alternative exhaust control mechanisms (engineering devices), as we would if we experimented with mixtures of SO_x and CO and NO_2 or whatever. Perhaps we would gain more information which could be extrapolated to arbitrary fuels or engine types, if we experiment with more basic components. I also don't believe in dose-response studies. Think how nice it would have been if we had had 50 animals in each group instead of 12. We would have something really definitive. We could quantitate incidence very closely. We could have done serial sacrifices. If we get into another experiment where we try to take a "birdshot" approach at 8 or 10 atmospheres, the money will have to be divided correspondingly, and what we really have is a very expensive mix of small studies. Each atmosphere by itself wouldn't constitute much of an experiment, but collectively they cost a great deal of money. So I would make it more simple and bigger. I'd use concentrations high enough so that the effects could be accelerated, so long as it's not so high that you get into a different mechanism for production of the effect. Retain the mechanism but accelerate the effects. I think the reason it isn't so important to simulate community atmospheres is that we don't regulate community atmospheres. We regulate the content of exhaust emission. There's a rather tenuous relationship and a very imperfect correlation between what comes out of the exhaust pipe and what we breathe anyway, so why is it so important that we simulate a community atmosphere? The important thing is to find out whether what comes out of the exhaust may produce certain types of effects.

Tyler: I would like to ask our consultants once more regarding design of future studies because we must soon bring the conference to a close.

Brain: It is important to define dose-response curves for our major pollutants and this requires a number of levels of exposure. I would also add that in addition to number of levels, a future design might also want to incorporate important interactions such as infection, smoking, occupational dusts, genetic susceptibility, very young and very old animals, and animals with pre-existing disease. Also, perhaps species differences can be exploited. For example, it might be useful to pick out the species which are both most sensitive and least sensitive. Let's look at the extremes of the bell-shaped curve and ask, what is responsible for those differences in susceptibility? Is it morphological? Is it physiological? Does it relate to life span? One man's noise can be another man's signal. Perhaps that might be an aspect of species differences that could be utilized in a useful way.

Kleinerman: We must be careful when we draw conclusions from studies at high concentrations and apply them to lower concentrations. We must prove that the consequences of these different exposures are similar before we can use different exposures interchangeably. I agree that if we can study the effects of single substances such as NO_2 or SO_2 or ozone, we will be farther ahead in understanding the mechanism of the lesions. The species of animal which we use is very important. The choice will depend in part on what one's goal is. If our goal is to try to understand mechanism, a small animal species may be as useful as any other provided that the desired experimental end point results. We can then manipulate the variables and observe the effects over a short time period. Important factors in utilizing a small animal species are its life span and the time necessary to create the end point.

McLees: I think there are a number of important things to glean from this sort of experiment biochemically and hemodynamically with respect to physiology and biochemical changes in the kinds of lesions that have been presented.

Tyler: You're speaking in favor of a mechanistic approach.

McLees: Well, finding out what's happening. Right.

Lewis: I take the opposite opinion of just about every point Mr. Busch raises. With the new technology in automobile engines, unleaded fuel, catalytic converters, and so on, the pollutant problems that we're dealing with are going to be different 10 years hence than they are now. This study has some relevance, but will not answer all the questions of the toxic hazards of automobile emissions from automobiles with catalytic mufflers and unleaded fuel. That's one thing. The other thing that Mr. Busch said is let's take it as it comes out of the tailpipe.

Busch: I didn't say that. You misunderstood me.

Lewis: Well, we need to cause a photochemical reaction to simulate the Los Angeles type of situation. It's going to be with us forever. Auto exhaust contains thousands of components. And, although this study with the internal combustion engine as we know it now relates to it qualitatively, it may not relate to it 10 years hence quantitatively. I do believe we want to try to approach dose-response studies, because if you don't have a "no-effect" level, the people that are setting standards have to do it somewhat arbitrarily. That's the key point. You're not going to be able to create synthetic atmospheres that either can be factorially removed from a complex mixture or necessarily relate to it. I think this study proves it on a biochemical response phenomenon, the differences between I versus NO_2-high. The known toxicologic phenomena of synergism, antagonism, and additive effect, all come into play on both a physical and a biological basis.

Busch: May I rebut that? Everything you say supports what I said. You just misunderstood me. You said that we won't know what we're going to have in the air 10 years from now, what kind of auto engine emissions control device we'll have. That's exactly the reason I thought we should use identifiable components, because if we can identify the toxicity of components, then we'll be able to make some extrapolation from the data we take today to some arbitrary or alternative engine emission control system. But if we experiment with a particular configuration now which contains unknown components, we won't be able to extrapolate from that to anything else. So I think what you said tends to be consistent with what I said.

MacEwen: I was a little startled at what Mr. Busch said. He is recommending the use of purer compounds or essentially identifiable compounds rather than complex mixtures. I understood him earlier to say he was opposed to dose-response studies, and I still definitely feel that it is the only way we are ever going to be able to extrapolate these data to other mixtures of fuels and to define the "no-effect" level for establishment of any kind of emission standards.

Busch: I said the "no-effect" level relates to a community atmosphere in which we breathe. None of us breathes diluted auto exhaust or irradiated auto exhaust. We breathe that plus all the industrial emissions and whatever comes from our home heating systems. I think we need to consider the whole community atmosphere and not just the auto exhaust emissions.

MacEwen: But I can define my community atmosphere in terms of SO_x or NO_2?

Busch: No, you can't. But you can probably make some inferences about the toxicity of the atmospheres if you know that certain components of it are toxic in themselves.

Malanchuk: The aerometry that was connected with this study was very considerable in spite of what some people might consider were just a few compounds being monitored. We had to address ourselves to doing what we did because of some limitation of instrumentation, people and time. Mr. Busch suggested limiting it to a couple of compounds, doing a chronic study over a long length of time, and using 50 animals instead of only 12. I must admit if I had been in that original committee back in 1965, I think I would have approved of their recommendations to eliminate several exposure atmospheres. If that had been done, where would we be today? So I think you have to spread it a little bit thin and try to avoid complete dependence on statistics. I was hoping that we could supplement statistics with a little bit of intuitive knowledge as to what direction it was taking.

Hueter: I just have a couple of remarks. First, I firmly believe it is the scientist's responsibility to assess the hazard of a pollutant, but it isn't his responsibility to set its risk. Times have changed since this study was planned in 1963-65. We are in a better position to identify what the components of these new energy technologies might be and to evaluate our screening methodologies from the standpoint of cytotoxicity. We can now better select the components of a mixture on which we should concentrate the toxicology and chronic studies. I really believe that we could learn more by not looking at the mixture but using the other disciplines that are available to us now ahead of time. So I support much of what Dr. Lewis said.

Tyler: You're supporting pure compounds?

Hueter: Simple mixtures of pure compounds. And I also firmly believe in mechanistic studies as long as they have a reasonable balance within the EPA laboratory with practical applied research, which the regulator needs. But for the long-range response, I think we have to study mechanism because mechanism might predict significant disease at some future date. One example of this is the infectivity model, which is the only mechanistic thing we've ever done, and it's very useful now.

Nettesheim: I would like to have a clarification, because I can't get the mixtures and the compounds straightened out. I heard Dr. Lewis emphasize the mixture as it is being produced, but I heard you emphasize a mixture of identified compounds. Is that correct? To me, that makes a big difference, and I think the latter goes pretty much along with what Mr. Busch said.

Hueter: I personally believe that if one has the proper chemical characterization of the effluent of interest, then, at the present state of toxicological knowledge and by using experts assembled to assess those identified compounds, you get further faster by looking at individual compounds that are given toxicity priority or simple mixtures of those compounds where you feel an interacting effect probably is going to occur, than you are by putting such an expensive facility to use with a mixture which can give you all kinds of interpretative problems.

Nettesheim: Yes, I think that's clear.

Tyler: Now I'm going to call for brief comments from people who were involved in the original study.

Lee: My support for chronic studies has been expressed by all previously, particularly by a fellow biochemist, Dr. McLees, so I'm not going to elaborate any further on the same point. But, I want to say a word about the support for the whole emission studies and single pollutant studies plus various combinations of single pollutants. I know this is probably going to sound like talking from both sides of the mouth, but without doing the whole mixture study, I don't think you can see the effects of interactions of certain compounds with the particulates and with other chemicals. So I think the whole mixture study is really necessary, and if I had one at this point, offhand, I would start with emissions from fossil fuel combustion because most of the sulfate, some 96%, is coming from stack emissions rather than auto emissions. For future studies, this is something that has not been brought up in this conference. I would also like to look at the nutritional influences. In many experiments, animals are overfed and overprotected, and in some cases, as Dr. Rouser mentioned, they may be deficient in certain essential nutrients. Another important point that I would like to mention is the provision of stable working atmosphere along with a stable funding mechanism rather than allowing some administrators or politicians to attack the study for its nonproductivity before its conclusion.

One other point that we should pay attention to is so-called positive results vs. negative results. Positive results in the conventional sense of basic research are important, however, negative results from well-conceived and well-run experiments could be just as significant in terms of public health.

Stara: What did this study show? Did it show that certain air pollutant mixtures produced from automobile exhaust caused definite emphysematous lesions? Is that what we can expect to occur in the human population? And if so, can these results be shown using short-term studies or even long-term studies of single pollutants? I believe that certain mixtures or major components of auto emissions together prove to be harmful and must be studied further, and perhaps the individual exhaust components as well. But without at least one group of animals being exposed to the actual total emissions, we couldn't develop an airtight case and obtain the results that we need. I also know that the auto industry, the power industry, and coal industry have projections into the future: they are now developing future technology and fields and so we could possibly predict at least some of the pollutants that may be associated with these processes. In some respects, we know what will occur in future years; therefore we could now plan the research on the effects of some pollutants that will be pertinent in the future. Hence if we started such a study now, we would have the data ready when they are needed. It would be a sort of "prospective" toxicologic research.

Tyler: Dr. Hueter, I'm going to call on you next. Then I'll call for comments from the people who were involved in the study but not with EPA, and then our consultants can make comments.

Hueter: I just had a couple of other comments. Dr. Lee talked about most of the sulfate coming from stacks and that's true, but that's probably a different kind of sulfate. That's aged sulfate aerosol. The catalyst is fresh, perhaps ultrafine sulfate, sulfuric acid, which is a little different problem. We're looking into that. Secondly, I think we've been a little short-sighted in that we're concentrating on just animal toxicity, and as far as EPA is concerned that isn't the only tool we have to look at health effects. We combine epidemiology, clinical human exposures, and animal toxicology. And when you're using all three disciplines to attack a problem, I think that puts a different perspective on the type of exposure that you're giving animals in a toxicological situation, because you have a possibility of verifying it in some instances with human exposures under controlled conditions and in other instances with correlative epidemiological findings.

Tyler: Thank you. I'm going to start with Dr. Dungworth, because he was the major contractor on the West Coast, as I understood it.

Dungworth: The only point I want to make is that it disturbs me to hear people talking about exposing animals to an ill-defined mixture of pollutants because it is impossible to sort out and analyze interactions within it. If we talk about defined mixtures, that is reasonable. If we want to know the interaction between particulates and oxidants and reductants we must use well-characterized exposures. Unless we know what the animals are breathing, and this is in the air, not necessarily the tissue dose, we are incapable of sorting it out. We have an oxide mixture that we have been discussing the last 2 days, the raw and irradiated automobile gasoline. And we have admitted that we can't sort out why apparently there was less damage in the raw and irradiated gasoline than there was with the purer compounds.

Bhatnagar: One of the things I'm concerned about is the fact that the study did not take into account a number of factors. For instance, human beings, when they are being exposed to this kind of exhaust, are not protected the way our dogs were. There were no social interactions, there was no possibility of catching a viral pneumonia which very likely would have been exacerbated simply by the fact of having been exposed. Then there were other factors such as nutrition which Dr. Lee has pointed out. We only used female dogs which were never allowed to be mated. I think these conditions should be taken into account in any kind of long-term study. Now, we've talked a great deal here about the realities of doing factorial types of experiments, whether we should look at a mixture or whether we should look at individual pollutants, and I really don't think this kind of thing can be solved very easily. We can't do a very large number of animals; obviously it's a very expensive operation. So we've got to find simpler, cheaper ways, and I think we have to think in terms of doing experiments using tissue cultures, organ cultures and other *in vitro* models.

Rouser: I would like to just add one thing which I discussed earlier. I think there is a very serious omission that doesn't have anything to do with biochemistry. It has to do with the developing organism. I think that development studies should start day one to try to assess what the effects are of agents both on the *in utero* development and the immediate postnatal period, where so much change takes place. I think that's something you should give some careful thought to.

Busch: I want to temper what I said before. I didn't mean to imply that we should use simple gases. Dr. Lee brought up the important point that it may be necessary for these gases to exert their effects to have particulates present, and if it's necessary to generate the kinds of particulates that are typical of the freeway and the city by using an engine, then that's a good reason to use an engine. But if you can get them in some better, simpler way so that they're more identifiable, then I would think you should. The best reason to use an engine is to be able to generate the types of identifiable components we want to examine.

Bhatnagar: I got the feeling that we as biochemists failed to provide useful information and useful input in this study. We got a very limited amount of data which do not explain the morphological or physiological changes. The reason for that perhaps is in the fact that our involvement was at the very terminal state whereas as a biochemist I know that the initial response may be the most important thing. For instance, we should have looked at the animals after the initial exposure to see if there was inflammation, if there were any kinds of changes in the whole biochemistry of the lung, or the whole organism. What kind of metabolic changes are occurring in different organs? What kinds of changes occurred in the tissues themselves? Lysosomes might have been mobilized to cause emphysema. So, in any future chronic study, I would like to recommend very strongly that a biochemical component should be included.

Stephens: I would like to take a brief look at the history of concern about health and air pollution. Originally (during the 1950's) much of the concern grew out of industrial problems but by the late 1950's epidemiologic studies had indicated that the air pollution and smoking were related to chronic diseases as well as cancer of the lung. A major criticism of early studies that exposed animals to whole cigarette smoke and smog was the lack of identity of the specific toxic agents within these complex gaseous and particulate mixtures. The major question was: what specific elements were harmful?

In the following decade or so there has been a marked increase in funding from NIH to study the problem and numerous studies on specific pollutants, such as sulfur dioxide, nitrogen dioxide, ozone, cadmium, and the like have been undertaken. A volume of data is now available to design and conduct a reasonable chronic investigation. The problem, as Dr. Dungworth put it, is what kind of a defined mixture can we handle and interpret. There are many people who can, and should, contribute to the design of any future chronic study because it will be extremely costly, and to date, chronic studies using low-level exposures do not have an impressive track record. It can probably be assumed, however, that regardless of the exposure regime agreed upon, there will be substantial criticism. If an exhaustive effort is made, however, to design the experiment well, fully utilizing the available data, then that is the best we can do.

There are only certain kinds of questions that can be answered with chronic studies, however they are important to the general public. I think the public needs to clearly understand the health risk involved in exposure to smog and other pollution. Chronic studies are tedious and it may be difficult to acquire the needed commitment on the part of qualified scientists to continue with such a protracted study.

Current information supports the usefulness of shorter exposures at higher levels and the reasonable extrapolation of such data to levels that currently exist in the environment. In addition, there is strong evidence that intermittent exposures evoke the same response as continuous exposures. Obviously there is a level of exposure to each toxic element that will overwhelm the animal and it will die after a brief period of exposure, usually under 48 hours. However, at only modestly lower levels the animal will adapt and survive for much longer periods giving the tissue time to respond.

In the case of ozone, it has become clear that levels between 0.2 and 1.0 ppm are useful for chronic studies of modest length (up to 1 year). Exposures to nitrogen dioxide from 1.0 ppm to perhaps as high as 20.0 ppm appear to be useful in terms of permitting various species to survive so that associated cellular and tissue responses can be studied and evaluated in terms of their health effects.

Mechanisms are obviously complex and involve biochemical, structural and physiological alterations that are progressive. While there are many things happening during the first few days, the insidious progression of the disease takes much longer. Protracted studies of several decades may be required to understand the details of various mechanisms involved and the use of simplified systems, such as protein gels for the study of free radical production resulting from nitrogen dioxide or ozone may be useful as demonstrated by Dr. Pryor.

For the past 2-1/2 years we have been working with postnatal animals, and their response was surprising in that they are remarkably resistant to both nitrogen dioxide and ozone. They provide a most interesting model.

MacEwen: There is one point I would like to make. It refers to the early set of cardiovascular studies that were done on these animals when they were still on the experiment. I think there is a report in the package concerning the measurements and electrocardiograms of the whole series of dogs which implies that there were some treatment effects, particularly in terms of right heart hypertrophy, some infarcts, and also widening of the QRS complex. I think that that should be repudiated at this point. I don't believe there were any changes. I don't think there is any evidence to substantiate it. Electrocardiographic changes, infarcts of any magnitude that would show up on randomly taken or periodically taken electrocardiograms, should have been of a sufficient magnitude to have been seen clinically or to have been observed at the necropsy. At least some scarring should have been visible if it was at that magnitude to have persisted over any period of time. However, none of these effects were seen twice in the same dog, as I understand. I don't think there is sufficient incidence of cardiovascular changes.

Littlefield: As far as the present study is concerned, it appears that the bulk of the tasks left are largely statistical in nature. What is needed to conduct a thorough statistical analysis of each end point?

Albert: I think this study provides a basis for thinking that there's real usefulness in a balanced, long-term program for chronic inhalation studies. It's obvious there needs to be a balanced mixture of dose-response studies, mechanism studies, single pollutant effects, interactions of different pollutants, species differences, etc. One thing that hasn't been mentioned here which I think is an important part is the intercomparison of human and animal populations. For example, the same quantitative morphologic studies could well be done on autopsy material on people living in cities with different levels of pollution. I think the immediate thing to do regarding this study is to finish the quantitative morphologic analyses on the rest of the dogs and on the excised lobes and get an overview paper written which would describe the characterization of the atmospheres and the functional and morphological effects and the interrelationships between the morphology and function. I think this would be very useful toward getting a long-term federal commitment toward the support of centers for chronic exposure studies.

Nettesheim: I agree with everything that has been said and I disagree with everything that has been said, which means that there is some justification for the various points of view that have been raised. I would like to turn that into a recommendation to the EPA. The thing that has kept me away from the EPA is the fact that they tend to avoid mechanism studies. I can see the need for the kind of study that has been done here, but I think in the long run, without support for basic research starting, we will run out of the tools and the knowledge needed to deal with the practical problems like the ones stemming from pollutants. I would say that any agency that expects the scientists to deal with the day-to-day problems must support basic research also. Therefore, I think it is wrong to permit a polarization between basic and applied research. We have to create a balance. The agencies must see to it that that is being done. Also, I do believe that what is being developed in carcinogenesis research is badly needed in toxicology. Namely, short-term assays and early indications. This may not always provide us with the complete answers but it will give us important leads. These assays may have biochemical end points or provide cellular end points in tissue culture, because anything that causes emphysema must have a cytotoxic effect at some time; if you find an agent is cytotoxic, it doesn't necessarily mean that it is an emphysematogenic agent, but if it is not cytotoxic it is not going to cause emphysema. I think the need for short-term assays is an important one and I think there needs to be a considerable amount of investment into the development of such assays. That's not a quick and easy thing to come up with. The National Cancer Institute has been supporting the development of *in vitro* carcinogenesis tests for the last several years, and it is anticipated that another few years are needed before we have the tests that are really needed. Lastly, I would like to say that I congratulate you on this conference. I think I have not been involved in anything like this before. I think it is a great idea to get all the people together that have been involved in such a complex study, plus a few others, to evaluate what has been accomplished at the end of a long and large experiment.

Tyler: Often the first indication that something has gone wrong in the health of the population is going to show up at the autopsy table. While I'm calling on Dr. Thurlbeck last, indeed many times pathology at autopsy is going to be the first indication.

Thurlbeck: Dr. Brain made a point which is critically important to the human pathologist, namely that one is often looking at interactions. The concept of modifying a long-term experiment to examine interactions with infectious and other agents is a critical one. I have no feeling about mixtures versus pure compounds other than that pure compounds sound much more logical. Sulfates and sulfuric acid seem to me a lot more important than I had previously recognized.

We have talked about advantages of using small animals because we can get quicker results. This is worth following. I'm suggesting that one might duplicate this experiment using small mammals and see if anything happens at the end of a couple of years. We may, however, be fooling ourselves in regarding chronicity in terms of lifetime of an animal. Lifetime and duration may turn out to be two different things. If lifetime and duration exposures are two different things, the human situation is very interesting. We have to think in terms of 50-, 60-, and 70-year exposures and extrapolate backwards to the longest possible exposure rather than the lifetime of an animal.

Two observations have surprised me. The chronic destructive lesion within the lung appears to be progressive after cessation of exposure, whereas intuitively it should be static. We are unclear as to whether the airway lesions are reversible, partially reversible, or progressive. They certainly cannot be totally reversible and I would have originally guessed that airway lesions would be reversible after cessation of exposure. This particular variable can be examined. I'd like to stress the point about the importance of mechanisms. Mechanisms are finally what it's all about. If we knew what the mechanisms were, then as changing technologies occur, as changing environments occur, one could then find rational solutions rather than make empirical decisions. Every applied agency has to recognize that it has to be looking at mechanisms at the same time.

I'd like to pick up the point about what we can find in the autopsy room. The available studies really have not addressed themselves properly to this problem. The reason is that the studies are extremely difficult. It would be useful to try to identify people who have lived in a non-urban environment for a long period of time and look at a whole variety of morphometric and functional variables on excised lungs. You can get a lot of good information out of excised lungs, and then compare these findings to the lungs of people who have lived in cities. The previous questions that have been asked are crude ones — these have been questions like, are there big holes in the lung? Are there big holes more frequently in the lungs of smokers than nonsmokers? Are these big holes more frequent in people who have lived in Montreal compared to Malmo in Sweden? These questions are crude ones, and we should be asking much more subtle ones like, is there loss of recoil in the lungs? Or are there subtle morphometric changes in dimensions of the lung in subjects who have lived in an urban environment? We should be asking, I think, whether there is evidence that lungs are prematurely and excessively aged because of environmental exposure.

Tyler: Thank you. I'm going to summarize the conference by saying that the experiment was much better planned than I had realized. It was conducted at a high level, and I think that it has shown significant results. I'd like to thank all of you for participating. With that, I'll turn it over to Dr. Stara.

Stara: I want to thank all of you for your input and interest; particularly for your willingness to work the long, early and late hours. I think this was an extremely hard-working and successful conference.

TECHNICAL REPORT DATA

(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA 600/8-80-014	2.	3. RECIPIENT'S ACCESSION NO.

4. TITLE AND SUBTITLE Long Term Effects of Air Pollutants: In Canine Species	5. REPORT DATE July 1980 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) J.F. Stara, J.G. Orthofer, D.L. Dungworth, W.S. Tyler	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS SAME AS BELOW	10. PROGRAM ELEMENT NO. A1LH1A 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS Environmental Criteria and Assessment Office Office of Research and Development U.S. Environmental Protection Agency Cincinnati, OH 45268	13. TYPE OF REPORT AND PERIOD COVERED Series 8 - (1970-1980) 14. SPONSORING AGENCY CODE

15. SUPPLEMENTARY NOTES

Research and Conference Proceedings Being Published Together

16. ABSTRACT

The Clean Air Act of 1970 as amended in 1977 requires that a comprehensive data base be established to assess human health effects caused by air pollution from mobile sources. The spectrum of potential toxic effects can be viewed from two perspectives: The first is the identification of toxic effects from combined low-level effects of the individual major ambient air pollutants, which are combustion by-products of automotive exhaust. Ideally, the major components of a data base used to develop health risk assessments are well-designed epidemiological studies and long-term, low-level animal studies.

The 9-year study presented in this monograph reviews the effects following exposure of dogs for 68 months to automotive exhaust, simulated smog, oxides of nitrogen, oxides of sulfur, and their combinations. Studies using canine species over extended periods of time have proven useful in the evaluation of risk to humans, especially when combined with epidemiological studies and human clinical investigations.

All of the data were reviewed at a conference held at Asilomar, California by invited expert scientists. Their evaluations and judgments form a significant segment of this monograph.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES 304
	20. SECURITY CLASS *(This page)*	22. PRICE

EPA Form 2220-1 (9-73)

☆ U.S. GOVERNMENT PRINTING OFFICE: 1980-660-934